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Growth, 1949, **13**, 1-6.

A PHENOTYPICAL DIFFERENCE IN GROWTH OF WILD RATS[1]

DAVID E. DAVIS

(Rec'd for publication December 2, 1948)

The growth of Brown Rats (*Rattus norvegicus*) in nature has not been studied although there is a vast literature concerning the growth of domesticated varieties (Dunn, 1947). The growth of wild rats is of interest for comparison with domesticated varieties, in addition to its inherent ecological value. The aim of this paper is to describe evidence concerning the source of differences in weight attained by wild Brown Rats belonging to different populations.

Differences in weight of individuals in wild populations have rarely been studied although growth of some species has been examined. Hacker and Pearson (1944) studied the changes in weight of the mouse *Apodemus sylvaticus* in England. In addition to determining the growth curve, the authors found that in the winter the small mice gained weight, the medium mice remained constant and the large mice lose weight. Other mammals have been studied only in captivity or for a short time after birth. Hamilton (1937) combined growth data for mice (*Microtus pennsylvanicus*) captured in the wild and raised in captivity to give a composite growth curve. This type of study, however, does not consider the problem of growth under different conditions.

A striking difference between the size of rats on a farm near Baltimore and in Baltimore city suggested an analysis of the reasons for such great differences. The city rats were trapped in the yards of residential blocks where there is usually an ample supply of food and harborage available. The farm rats were obtained on a farm which had 3 large barns and a corn crib. Stalls for race horses were built in all the barns and next to the crib. About 50 race horses, 10-20 pigs,

[1]Contribution from the Rodent Ecology Project of the Department of Parasitology, The Johns Hopkins School of Hygiene and Public Health. The work reported in this paper was conducted under a grant from the International Health Division of the Rockefeller Foundation.

10-12 cows and a miscellaneous assortment of chickens and ducks inhabited the farm. Because of ample food and harborage a large number of rats lived in all the buildings. The food consisted of horse feed, manure, and an occasional dead chicken or duck. Chicken feed was available occasionally.

The weights of rats caught in the city and of rats caught on the farm are compared in table 1. The differences between the means

TABLE 1

COMPARISON OF WEIGHTS OF FARM AND CITY RATS

Weight	Males		Females	
	Farm	City	Farm	City
0-24	11	8	4	9
25-49	101	76	101	65
50-74	87	131	108	122
75-99	53	120	104	105
100-124	80	81	81	80
125-149	46	58	77	78
150-174	63	66	97	77
175-199	45	45	83	67
200-224	52	59	109	86
225-249	52	61	69	69
250-274	60	50	87	84
275-299	80	59	92	83
300-324	85	53	72	69
325-349	62	76	41	45
350-374	59	66	16	92
375-399	37	67	19	79
400-424	28	65	2	94
425-449	10	65	2	86
450-474	1	70	0	59
475-499	0	48		57
500-524		43		36
525-549		25		14
550-574		22		9
575-599		13		8
600-624		3		3
625-649		2		2
650-674		0		0
Totals	1012	1432	1164	1578
Mean	207.8	263.6	182.6	261.1
S. D.	119.1	160.0	96.2	148.0

of farm and of city rats are significant ($P = .000$) for both sexes. The food of the rats in the city was largely garbage and apparently was an excellent diet because Dunn, et al. (1947:78) found that 640 gms. was the limiting weight for males of the largest strain (Yale

-Albino) discussed by them. The albino females however never attained the high weights of wild females.

Since it is obvious that the city rats are larger than the farm rats, some experiments were undertaken in the laboratory to determine if diet was a factor in the difference. Young rats weighing 50-100 gms. and probably 6-10 weeks old, were captured in the city and on the farm. They were then kept in laboratory cages measuring 45 x 60 x 30 cm., with a mesh wire floor. At first 10 rats kept in a cage but after the rats became half grown no more than 5 were kept in 1 cage. Water was always available from a glass bottle and food was kept in partially closed metal dishes.

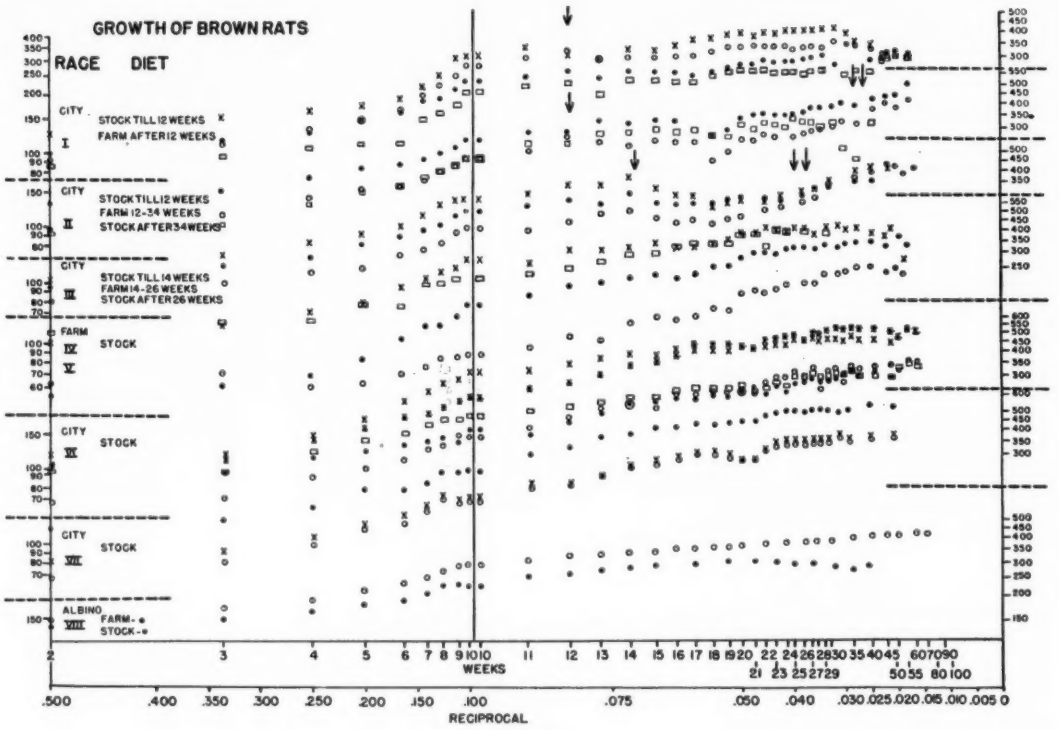
The McCollum stock diet consists of: Wheat (soft) 30.0%, maize (yellow) 20.0%, rolled oats 20.0%, casein (crude) 3.5%, whole milk powder 25.0%, $CaCO_3$ 0.5%, ferric citrate .0011%, $CuSO_4.5H_20$ .0004%, NaCl 1.0%, and fresh greens (cabbage, kale, spinach, etc.) three times weekly.

The farm diet consisted of a commercial horse feed, (oats, corn, alfalfa meal, "Peet" minerals, and dried milk), fresh manure several times a week, and an occasional dead rat.

The history of the groups of rats and their diets are given in table 2. The growth of the male rats are plotted in the figure on semi-logarithm paper, using Zucker's method (Dunn, et al., 1947). To be sure that the McCollum diet was not deficient, it was supplemented

TABLE 2
DIETS OF FARM AND CITY RATS IN LABORATORY

Group	Source	Males	Females	Diet	Date for Start of Diets
I	City	5	5	McCollum Stock + 10% Liver	Nov. 21, 1946
		4	5	Farm Diet	Feb. 10, 1947
II	City	4	4	McCollum Stock + 10% Whey Powder	Nov. 13, 1946
		4	4	Farm Diet	Feb. 5, 1947
		4	4	McCollum Stock	July 2, 1947
III	City	5	5	McCollum Stock + 10% Soybeans	Nov. 4, 1946
		3	4	Farm Diet	Feb. 10, 1947
		3	2	McCollum Stock	May 15, 1947
IV	Farm	3	2	McCollum Stock	Nov. 18, 1946
V	Farm	1	4	McCollum Stock + 10% Corn Germ	Dec. 23, 1947
VI	City	5	5	McCollum Stock	Oct. 10, 1946
VII	City	5	5	McCollum Stock + 10% Corn Germ	Oct. 23, 1946
VIII	McCollum	10	0	Farm Diet	Feb. 10, 1947



FIGURE

Growth of rats plotted on semilogarithm paper by weight in grams against reciprocal of time in weeks. See table 2 and text for explanation of diets.

with liver, whey powder, soybeans, or corn germ, groups I, II, III, VII). Since there was no apparent difference in the early growth of these groups, it was concluded that the McCollum diet was adequate. It will be noted that the 4 male rats of the farm strain and the 8 male rats of the city strain grew at about the same rate on stock diet. The city rats when changed from McCollum stock diet to farm diet (indicated by single arrow) lost weight or grew at a slow rate for many weeks. When returned to the McCollum diet (double arrows) the city rats grew more rapidly. The albino rats on farm diet grew more slowly than the albino rats on McCollum stock diet. From these data it is clear that the diet on the farm was deficient in some way. It is not within the scope of this work to determine what the deficiency is but merely that the diet is largely responsible for the difference in growth rate of the rats from 2 different places.

It is further of interest to compare the weight of the farm rats living in the barns with the weight of rats living in the corn crib where corn was frequently but not always available. The crib males (230.6 ± 87.5 gms.) were significantly ($P = .000$) larger than the other males (197.0 ± 117.2 gms.). The crib females (203.2 ± 85.0 gms.) were also significantly ($P = .000$) larger than the other females (176.2 ± 98.0 gms.).

This paper does not pretend to analyze the differences in growth in detail. It is fully realized that some genotypical differences may exist and that psychological factors may be important both in the wild and in the cages. The data here presented support the conclusion that the differences in weight of city and farm rats was principally phenotypical being the result of differences in diet.

Summary

A comparison of the weights of wild rats caught on a farm and in Baltimore showed that the city rats were significantly heavier. Rats from the farm and the city grew at approximately the same rate on the McCollum diet. Rats from the city grew slowly on the farm diet. It is concluded that dietary differences are largely responsible for the differences in weights of the 2 populations of rats.

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I am greatly indebted to Ernestine Becker McCollum for advice in planning the diets.

Growth, 1949, **13**, 7-20.

THE RELATION BETWEEN PHOTOMETRIC TURBIDITY AND BACTERIAL CONCENTRATION (BACTERIMETRIC STUDIES. IV.)*

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(Received for publication Mar. 25, 1949)

The modern photoelectric spectrophotometer permits speedy and precise measurements of bacterial growth. Results are directly obtained in terms of such functions of bacterial concentration as galvanometer reading, transmission or extinction percentage, or optical density. For the evaluation of quantitative aspects of growth, e.g. growth rate or proportionality of response to limiting nutrients, measurements must be converted into direct expressions of bacterial quantity, such as number of organisms, dry weight or bacterial nitrogen. Calibration of optical readings in terms of such mass units, performed by many authors for specific purposes, is laborious and of limited precision.

However, frequently absolute magnitudes are not required and the necessary information will be conveyed by relative figures of adequate accuracy which are directly proportional to bacterial quantity. Optical density, defined as the negative logarithm of transmission percentage, is directly proportional to the concentration of light absorbing material whenever each particle absorbs the same fraction of the impinging light regardless of concentration (Beer's law). Generally, this holds true for solutions but, because of the role of scattered light, not for suspensions.

Fig. 1 gives an example of how relative bacterial concentration, optical density and transmission percentage (or galvanometer reading) are related. It shows that a tenfold increase of concentration is reflected in a less than eightfold increase of optical density and in an approximately fourfold increase of extinction percentage. Obvious-

*Aided by a grant from Mary Curtis Zimbalist.

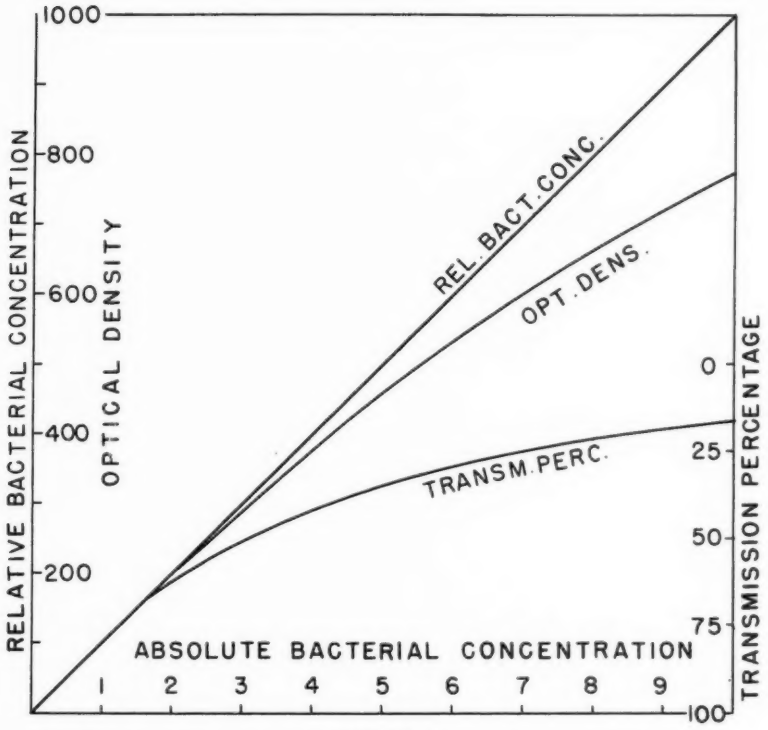

FIGURE 1

RELATIONS BETWEEN RELATIVE BACTERIAL CONCENTRATION, OPTICAL DENSITY AND TRANSMISSION PERCENTAGE

The relation between concentration and density is based on data of this paper. Scales are arranged so that the plotted values for the readings of the first tenth of the concentration scale coincide.

ly, for all but very low bacterial concentrations, graphs plotted on an arithmetic scale of transmission, extinction or galvanometer units, represent the true relations with great distortion. When optical density is used as the scale unit correspondence to reality is closer, the degree of distortion depending on, and expressing deviation from, Beer's law.

It has been pointed out (Mestre, 1935; Dognon, 1943, 1945) that the specific optical density of suspensions may be expected to decrease with increasing concentration because of the increasing relative contribution of scattered light to the total transmission. While the effects of such variables as size and transparence of particles, wavelength, and distance between absorbing medium and optical receptor preclude a precise theoretical treatment, the actual relations existing in particular systems of measurement may be established empirically.

In a study of such relations by Longsworth (1936) the optical densities of serially diluted water suspensions of a single bacterial organism were measured in a photoelectric instrument of the author's own design, and with white light. The expression derived from these measurements (equation 11, l.c) may be rewritten as

$$AOD = 2000 - 100\sqrt{400 - 0.4\ NOD} \qquad (1)$$

where NOD represents the observed net optical density values ($1000 \times \log Io/I$; cf. Toennies and Gallant, 1948) and AOD (adjusted optical density) the derived ideal linear values.

In the present paper data are presented for seven species of lactic acid bacteria, on the relations between optical density and bacterial concentration. The cultures were grown and measured in different media. The determinations were made in Coleman spectrophotometers at 675 mμ, and in round tubes of different diameters. It has been found that under the variety of conditions studied net optical density values up to approximately 800 can be converted into ideal linear values, i.e. relative bacterial concentrations, by the equation

$$NOD = AOD - (0.01\ AOD)^2 - 2(0.01\ AOD - 2)^2 \qquad (2)$$

In this equation the last member is omitted when $0.01\ AOD - 2$ is negative, so that the formulas

$$AOD = 5000 - 100\sqrt{2500 - NOD} \qquad (3)$$

and

$$AOD = 1800 - 100\sqrt{321.33 - 0.33\ NOD} \qquad (4)$$

result, for NOD values up to and above 196, respectively.

The practical value, in bacterimetric work, of an adjustment formula of broad validity is discussed below.

EXPERIMENTAL

General.—The data were obtained by measuring serial quantities (1 to 10 cc, 1 to 6 cc, or 0.1 to 1.0 cc) of a suitable bacterial suspension into appropriate tubes, completing volumes (to 10, 6, or 1 cc) with a clear medium, and reading the optical densities of the resulting suspensions and proper controls. The general procedure, including sterilization and photometry, was as previously described by us (Toennies and Gallant, 1948). However, all readings were taken at 675 mμ. This wave band is now being used because the absorption minimum of the color of our synthetic medium (1.c) is at 675 — 680 mμ and the net optical densities of suspensions, obtained by deducting the reading of non-inoculated tubes, are approximately 3 per cent higher than at the previously used 700 mμ level. For measurements with 100 x 10 mm culture tubes a concentric adapter[1] inserted into the hole of the Coleman cuvette carrier was used. The window of the adapter facing the light source is a 2½ x 6 mm rectangle.

Only non-limited[2] media were used in this work. 0.01 per cent of Merthiolate[3] was added to cultures and dilution media. This concentration of the germicide arrests actively growing cultures of the organisms used within 10 minutes to two hours. Unless otherwise stated, dilution media consisted of uninoculated but sterilized and incubated culture media.

Organisms and Media.—The seven organisms studied are *Lactobacillus arabinosus 8014, Lactobacillus brevis 8257, Lactobacillus casei 7469, Lactobacillus fermenti 9338, Leuconostoc mesenteroides P-60, 8042, Streptococcus faecalis 9790,* and *Streptococcus faecalis 8043* (*Streptococcus lactis R*). Four different media have been employed, as follows:—

A. This is our previously described (1.c.) synthetic amino acid medium, characterized by the presence of nucleic acid hydrolysate and 0.30 *M* phosphate of pH 6.5.

B. This medium was used only for resuspension purposes (Table 1). It is a colorless modification of medium A, obtained by omitting

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[1]Designed by H. M. Winegard.

[2]Media which contain all of their components in maximal amounts; *cf.* footnote of paper I of this series (Toennies and Gallant, 1948).

[3]We are indebted to Dr. A. Lee Caldwell of the Lilly Research Laboratories for the crystalline compound.

sterilization and incubation, and leaving out riboflavin and nucleic acid hydrolysate.

C. A semi-synthetic medium which we found advantageous for growing inocula for folic acid determinations (Toennies and Gallant, 1949). It contains, per liter, 20 gms bacto-casitone, 5 gms bacto-peptone, 20 gms anh. sodium acetate, 20 gms glucose, 2.5 gms each of KH_2PO_4 and K_2HPO_4, 200 mg $MgSO_4 \cdot 7H_2O$ and 10 mg each of NaCl, $FeSO_4 \cdot 7H_2O$ and $MnSO_4 \cdot 4H_2O$. It is filtered and sterilized 5 to 10 minutes.

D. A synthetic hydrolysate medium used by us in a study of the metabolism of pteroyltriglutamic acid (Toennies and Gallant, 1949). It is based on the *L. casei* medium of Teply and Elvehjem (1945), differing from the latter in that vitamins (using pyridoxamine) and phosphates are present according to the specifications of Roberts and Snell (1946), and in that casein hydrolysate and peptone are replaced by the "vitamin-free" enzymatic casein hydrolysate of the Nutritional Biochemicals Corporation (Cleveland 5), in an amount of 0.12 cc per cc of finished medium.

Results. Table 1 to 3.—One objective of the measurements summarized in Table 1 was to examine the possible role of culture conditions, with regard to optical and chemical properties of the bacteria. Therefore one crop (series I) was incubated for 17 hours at 29°, another (series II) for 24 hours at 42°, and a third (series III and IV) for 7½ hours at 42°. The organisms used for series IV were, like those of series I to III, grown in medium A, but for the measurements were washed and resuspended in the colorless medium B.

The observed optical density values, recorded as NOD, i.e. after subtraction of the optical density of the medium, are listed only in the first example (series I) because the "AOD found" values are directly derived from the NOD values (equations (2) to (4) and Table 8). Series II, III, and IV contain several values exceeding AOD 1000. The conversion formula used in these cases is

$$\text{NOD} = \text{AOD} - (0.01\ \text{AOD})^2 - 2(0.01\ \text{AOD} - 2)^2 + 2(0.01\ \text{AOD} - 10)^2 \qquad (5)$$

The additional term, compared with (2), (3) and (4), applies only when 0.01 AOD — 10 is positive, i.e. for NOD values above 767 (AOD = 1000). In practical work such values should be avoided because of their inherent inaccuracy. The "ideal unit values" (AOD

per cc) shown in the Table are obtained as follows. Plot the values of NOD per cc against cc, and by graphic extrapolation to 0 cc determine the approximate value for the slope of origin of the NOD curve. Use this provisional unit value for the calculation of provisional AOD values for the 10 concentrations. Calculate the mean absolute percentage deviation of the experimental AOD values from the calculated AOD values. On the basis of this deviation increase or decrease the provisional unit value until an optimal fit is obtained.

Duplicate determinations[4] showed that suspension series I (Table 1) contained, per 100 AOD units per 10 cc, measured in tubes of

TABLE 1

RELATIVE BACTERIAL CONCENTRATION AND OPTICAL DENSITY, S. FAECALIS 9790

Series			I		II		III		IV	
Suspension medium			A		A		A		B	
O. D. of suspension medium	82				83		32		6	
Amt. of bacterial suspension p. 10 cc.	NOD		AOD		AOD		AOD		AOD	
cc	fd.	p.cc	fd.	calc.	fd.	calc.	fd.	calc.	fd.	calc.
1	86	86	87	86	151	147	122	118	111	107
2	168	84	171	172	297	295	235	236	223	214
3	244	81	250	258	442	442	352	353	333	321
4	326	82	341	344	589	589	468	471	437	428
5	404	81	433	430	718	737	576	589	529	535
6	476	79	524	516	866	884	699	707	626	641
7	530	76	597	602	1042	1031	817	825	728	748
8	592	74	687	688	1184	1178	934	942	845	855
9	656	73	786	774	1324	1326	1083	1060	934	962
10	698	70	859	860	1474	1473	1187	1178	1025	1069
Ideal unit value, AOD p. cc				86.0		147.3		117.8		106.9
AOD values found: mean absolute deviation from calculated values			—0.1%		0.0%		0.0%		0.0%	
AOD values found: average deviation from calculated values			±1.1%		±1.0%		±1.3%		±2.7%	

16 mm inner diameter, 336 micrograms of dry substance, 36 micrograms of nitrogen and 10.3 micrograms of phosphorus, while suspension series II contained 392, 45 and 11.5 micrograms, respectively. Presumably, the "older" culture (II) contains somewhat smaller organisms, but their N and P contents (11.5 and 2.9 per cent) differ little from those of culture A (10.7 and 3.1 per cent).

[4]We are indebted to Mr. J. J. Kolb for these analyses.

Tables 2 and 3 show studies of graded suspensions of six additional organisms.

Relation between readings obtained in tubes of different size.— Identical suspensions were read in three types of tubes, (a) the

TABLE 2

RELATIVE BACTERIAL CONCENTRATION AND OPTICAL DENSITY. MEDIUM C.

Organism	L. CASEI				S. LACTIS R.			
Total volume	10 cc		1 cc*		10 cc		1 cc*	
O. D. of medium	67		24		67		24	
Volume fraction of bacterial suspension	AOD fd.	calc.	AOD fd.	calc.	AOD fd.	calc.	AOD fd.	calc.
0.1	98	90	90	97	93	84	96	97
0.2	184	180	186	194	178	168	194	194
0.3	267	270	304	291	255	252	288	292
0.4	352	360	392	388	330	336	387	389
0.5	444	450	477	485	425	420	488	486
0.6	532	540	568	582	499	504	575	583
0.7	624	630	681	679	557	588	663	680
0.8	696	720	770	776	640	672	804	778
0.9	807	810	900	873	735	756	876	875
1.0	904	900	1025	970	817	840	1004	972
AOD values found, av. deviation	±2.2%		±3.2%		±3.7%		±1.4%	

*Measured in 100 x 10 mm. culture tubes.

TABLE 3

RELATIVE BACTERIAL CONCENTRATION AND OPTICAL DENSITY. MEDIUM C.

	L. MESENTEROIDES		L. ARABINOSUS		L. BREVIS		L. FERMENTI	
O. D of medium	61		56		83		95	
Amt. of bacterial suspension per 10 cc	AOD		AOD		AOD		AOD	
cc	fd.	calc.	fd.	calc.	fd.	calc.	fd.	calc.
1	108	101	106	96	103	98	63	63
2	203	203	197	193	201	196	128	125
3	308	304	292	289	288	294	190	188
4	405	406	380	386	379	392	254	251
5	512	507	468	482	485	490	312	313
6	602	608	565	578	586	588	374	376
7	708	710	661	675	678	686	434	439
8	798	811	754	771	796	784	502	502
9	896	913	854	868	867	882	555	565
10	974	1014	950	964	980	980	624	627
AOD values found, av. deviation	±1.8%		±2.7%		±1.9%		±0.9%	

regular Coleman cuvettes, 19 x 150 mm, selected, (b) Pyrex culture tubes, 16 x 150 mm, and (c) culture tubes, Arthur H. Thomas Co. Cat. No. 9446, 10 x 100 mm. According to calibrations the optical path lengths of the (a) tubes cover a range of approximately 2 and those of the (c) tubes approximately 4 per cent. The (b) tubes were unselected. Table 4 summarizes the measurements. The ratios

TABLE 4
BACTERIAL CONCENTRATION, TUBE SIZE AND OPTICAL DENSITY. MEDIUM C.

	Bacterial suspension per 10 cc total volume cc	Large tubes (a) AOD*	Medium tubes (b) AOD*	Small tubes (c) AOD*
L. Casei				
	10	2380	1980	1295
	5	1175	990	624
	3	684	606	365
S. Lactis R				
	10	2350	1830	1275
	5	1130	986	609
	3	656	565	349

*Using equation (5) for AOD $>$ 1000.

of the AOD values obtained in tubes of different type are essentially constant: 0.85 $\pm$ 0.03 between medium and large tubes, and 0.535 $\pm$ 0.005 between small and large ones. Caliper measurements of the mouths of the tubes showed that these ratios correspond to the ratios of the internal diameters.

The results confirm the practical validity of formula (5) for these suspensions and indicate that adjusted optical density is proportional to path length (Lambert's law), thus permitting interconversion of readings obtained in tubes of different size, with the aid of optically determined diameter ratios.

Table 5 shows three series in which the same suspensions were measured in both large and small tubes. Because in these series (medium D) large deviations from equation (5) were encountered for readings corresponding to AOD $>$ 1000 only the results covering the range of equations (2) to (4) have been recorded.

Comparison of optical instruments.—In view of the role of instrumental factors, such as distance between absorbing medium and optical receptor, in determining the opacity of suspensions (Mestre, Dognon, l.c.), a comparison was made, on two series of suspensions, between the readings obtained in the Coleman model 11 spectrophoto-

TABLE 5
RELATIVE BACTERIAL CONCENTRATION AND OPTICAL DENSITY. MEDIUM D.

Amount of bacterial suspension per 6 cc*	L. CASEI		S. LACTIS R		L. MESENTEROIDES	
	AOD		AOD		AOD	
cc	fd	calc.	fd.	calc.	fd.	calc.
0.50			173	173		
(0.54)	96	99	178	187	79	74
1.00			341	347	138	138
1.00	186	186	343	346		
(1.07)	197	198	349	371	153	148
(1.07)	201	198				
1.50			511	519		
(1.61)	281	299	571	557	226	223
2.00	360	372	705	693	268	277
2.00	371	372	684	693		
(2.14)	394	398	772	742	297	296
(2.14)	391	398				
2.50			925	866		
(2.68)	486	497			370	371
3.00	564	557			417	415
(3.21)	627	596				
(3.21)	593	596			423	444
4.00	765	744			555	553
4.00	754	744				
(4.28)	830	796				
5.00	954	929			687	691
6.00					805	829
AOD values found, av. deviation	±2.2%		±2.8%		±2.0%	

*Parentheses indicate determinations made in small tubes. The actual concentration values have been converted by assuming that for equal AOD values concentration is inversely proportional to tube diameter; *e.g.*, the optical density of a 1 : 6 suspension measured in a small (c) tube corresponds to that of a 0.535 : 6 suspension measured in a large tube.

meter and in the more recent model 14. At the same time, to include data on another type of instrument, comparison was extended to the Klett-Summerson photoelectric colorimeter. A red filter (A No. 25) which transmits light above 600 mμ was used. The internal diameter of the tubes used in this instrument is approximately 80% of that of the Coleman tubes.

The results, shown in Table 6, indicate that the readings of the two Coleman instruments differ slightly, both as regards the color effect of the medium and the turbidity effect. However, in each suspension series the ratio of the AOD values is essentially constant so that the proposed adjustment system appears valid for the model 14 as well as the model 11 apparatus.

TABLE 6

COMPARISON OF BACTERIAL TURBIDITY MEASUREMENTS OBTAINED WITH DIFFERENT INSTRUMENTS*

Organism	L. BREVIS				L. MESENTEROIDES			
Instrument	Colem. 11	Colem. 14	Colem. 11	Klett-S.	Colem. 11	Colem. 14	Colem. 11	Klett-S.
O. D. of medium	90	95	89	40	61	75	61	28
	AOD	AOD	AOD	NOD	AOD	AOD	AOD	NOD
	101	102	101	11	105	101	103	10
	204	203	197	25	188	180	185	22
	292	291	288	36	290	290	279	32
	382	377	375	48	381	372	370	40
	488	484	481	60	489	471	458	52
	586	579	582	73	563	541	539	65
	671	663	672	83	668	642	639	74
	791	780	786	91	762	737	726	82
	860	839	856	102	845	816	812	93
	956	946	948	116	934	908	891	101
Av. percentage of Coleman 11 readings		99.0±0.7		12.2±0.4		96.9±0.8		11.3±0.4

*The suspensions used had stood for different periods of time. Because of evidence of secondary changes in bacterial concentration the readings have no significance with regard to linearity (vertical columns).

When allowance is made for the difference in tube diameters the turbidity readings of the Klett-Summerson instrument are only 15 per cent of those of the Coleman. It is well known that bacterial turbidity effect decreases with increasing wave length, and the low opacity measurements of the Klett-Summerson instrument seem attributable to the transparency of the bacteria to the long wave lengths transmitted by the glass filter. Measurements made under such conditions would be useful for the analysis of very dense bacterial suspensions such as occur in non-limited media.

Validity of adjustment formula at different wave lengths.—In two series of suspensions a comparison was made between readings obtained at 600, 675, and 750 mμ. The results, presented in Table 7, indi-

TABLE 7
COMPARISON OF BACTERIAL TURBIDITY MEASUREMENTS MADE AT DIFFERENT WAVE LENGTHS*

Organism	L. BREVIS				L. ARABINOSUS			
Wave length, mμ	675	600	750		675	600	750	
O. D. of medium	90	173	89		56	111	75	
	AOD	$\frac{AOD_{600}}{AOD_{675}}$	$\frac{AOD_{750}}{AOD_{675}}$	$\frac{AOD_{600}}{AOD_{750}}$	AOD	$\frac{AOD_{600}}{AOD_{675}}$	$\frac{AOD_{750}}{AOD_{675}}$	$\frac{AOD_{600}}{AOD_{750}}$
	101	1.287	0.950	1.355	106	1.204	0.868	1.391
	204	1.181	0.863	1.369	197	1.168	0.914	1.278
	292	1.151	0.870	1.323	292	1.144	0.890	1.285
	382	1.167	0.872	1.339	380	1.155	0.840	1.376
	488	1.158	0.855	1.355	468	1.152	0.853	1.351
	586	1.147	0.843	1.360	565	1.149	0.860	1.336
	671	1.151	0.842	1.366	661	1.136	0.870	1.306
	791	1.140	0.844	1.350	754	1.130	0.847	1.333
	860	1.128	0.835	1.351	854	1.129	0.856	1.319
	956	1.124	0.828	1.358	950	1.113	0.838	1.328

*Cf. footnote to Table 6.

cate that with increasing values of AOD_{675} the ratios of AOD_{600}: AOD_{675}, as well as the ratios AOD_{750}: AOD_{675}, tend to decrease while the ratios of AOD_{600}: AOD_{750} tend to be constant, thus suggesting that at 675 mμ, the wave length selected because of minimum color effects at that point, the deviations from Beer's law of the bacterial suspensions are also at a minimum.

Application and Discussion.—A convenient adjustment table covering all NOD values up to approximately 800 (AOD 1000), may be obtained from calculations and graphic interpolations. A skeleton of

TABLE 8

OPTICAL DENSITY ADJUSTMENT TABLE FOR MEASUREMENTS IN THE COLEMAN SPECTROPHOTOMETER OF SUSPENSIONS OF LACTIC ACID BACTERIA

The AOD values are shown inside the heavy lines; *e.g.*, an NOD value of 550 corresponds to an AOD value of 626.

NOD	0	10	20	30	40	50	60	70	80	90
0	0	10	20	30	40	50	60	71	81	91
100	101	112	122	132	142	153	162	173	184	194
200	204	215	225	236	246	257	267	278	289	300
300	311	322	334	345	357	368	380	392	404	416
400	428	440	452	465	477	490	503	516	529	543
500	556	569	583	597	612	626	640	655	669	684
600	699	714	729	745	760	777	793	810	828	845
700	862	880	898	917	936	956	976	996	1017	1038

the complete Table in use in this laboratory is shown in Table 8. Two examples may be given for the practical usefulness of the complete adjustment table. First, the practical advantages of linear dose-response relationships in bacterimetric analysis are obvious and their mathematical aspects have been discussed by Wood and Finney (1946). Adjustment of observed optical densities for deviations from Beer's law will disclose the existence of linear relationships which would not be evident on an unadjusted optical density or extinction percentage basis. Secondly, adjustment of photometric turbidity values is necessary whenever rates of bacterial growth or lysis (e.g. Toennies and Gallant, 1949, J. Biol. Chem.) are being studied in quantitative terms. In a study of the division rates of several organisms in different media (unpublished), conversion of observed optical densities into adjusted values has given well-defined linear relationships when logarithms of AOD values are plotted against time.

The data here presented are not held to support the inference that the proposed formulas express the actual relations for different organisms and different media with absolute or with uniform precision. They are thought to demonstrate that the suggested procedure can convert a non-proportional function of bacterial concentration, viz., optical density, into another function which is linearly related to concentrations within limits of accuracy which are approximately those of the precision of the measurements themselves. The adjusted values, being mathematical functions of the observed values, are no less precise

than the latter. However, situations where during the course of bacterial proliferation, size or composition of bacteria change sufficiently to be reflected in the optical properties, obviously are not covered by the present measurements.

Summary

A study has been made of the optical densities of serial dilutions of seven species of lactic acid bacteria. The study involved four different culture media, and each culture was diluted with its own medium. The optical densities were measured at 675 mμ, in two types of Coleman spectrophotometers and in round tubes of different diameters. It was found that in all cases adequate corrections for the deviations of the optical densities from Beer's law can be made by application of the formulas

$$\text{AOD} = 5000 - 100\sqrt{2500 - \text{NOD}} \qquad (3)$$

and

$$\text{AOD} = 1800 - 100\sqrt{321.33 - 0.33\,\text{NOD}} \qquad (4)$$

for NOD values up to 196, and from 197 to about 800, respectively. AOD signifies adjusted optical density, i.e. a linear function of bacterial concentration, and NOD signifies net optical density, i.e. observed optical density minus optical density of the suspension medium. Optical densities are expressed in units which are 1000 times the logarithms of the intensity ratios of incident to transmitted light.

The AOD value exceeds the NOD value by 1 per cent at NOD 100 and, gradually increasing, by 32 per cent at NOD 800.

Correction of actual readings by means of an adjustment table based on the proposed formulas, yielded AOD values, which for 15 dilution series, covering approximately 150 readings, differed in the average by ± 1 per cent from exact linear values.

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INDIVIDUAL CHARACTERISTICS OF THE BACTERIAL GROWTH RESPONSES INDUCED BY DIFFERENT AMINO ACIDS (BACTERIMETRIC STUDIES. V.)*

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We have described (4) a medium for bacterimetric studies with *Streptococcus faecalis* (A.T.C.C. No. 9790) which, apart from the usual concentrations of glucose, salts, amino acids and vitamins and the presence of a nucleic acid hydrolysate, is characterized by a high concentration (0.30 *M*) of phosphate buffer of pH 6.5. The aim of the latter modification was to increase the range of linearity of the growth responses induced by limiting concentrations of amino acids. Observations have now been made on the responses induced by the eight amino acids for which Stokes (2) found *S. faecalis* to be a suitable assay organism.

The conditions of sterilization, incubation and turbidimetric measurement were those previously (4) described. However, readings were taken at 675 mμ and corrected for deviations from Beer's law (6). The amino acids used were *L*-threonine, *DL*-isoleucine, *DL*-valine, *DL*-methionine, *L*-arginine, *L*-histidine and *L*-lysine. The *D*-compounds present are assumed to have no effect (2). Response to each amino acid was determined at five different concentration levels (including zero) and in duplicates. Readings were taken after 22, 44, and 66 hours of incubation. The average difference between the readings of duplicates was 4 per cent. The results of this series of experiments are shown in the individual graphs of Fig. 1. For reasons of simplicity not more than two points are marked on each curve, namely those corresponding to the quantities of amino acid which at the time of the 22-hour reading gave adjusted optical density (AOD, rf.6) values of 300 and 600 (with the exception of lysine; see below).

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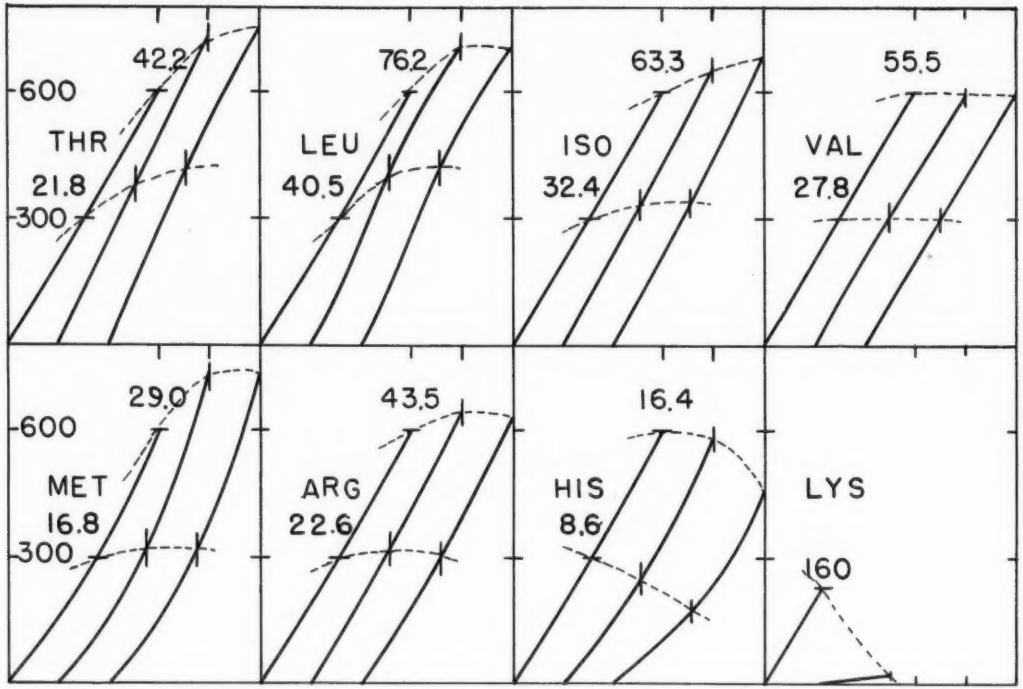

FIGURE 1

Growth responses to individual limiting amino acids in the provisional buffered medium. For each individual curve the abscissa gives the quantities of the respective *L*-compound (per 10 cc of otherwise complete medium) and the ordinates the adjusted optical densities (AOD, rf. 6). In each case the three lines from left to right show the growth responses after 22, 44, and 66 hours of growth (at 37.7° water bath) respectively. For the 44 and 66-hour curves the abscissa scales have been shifted by equal distances to the right, so that in each case the starting point on the abscissa represents zero micrograms of the limiting amino acid. The numerals shown on the 22-hour curve indicate the number of micrograms of *L*-compound which at that time produce bacterial growth corresponding to AOD values of 300 and 600, respectively. The AOD values subsequently attained by these two amino acid levels are marked by vertical bars on the 44-hour and 66-hour curves. Rapid disappearance of initial growth in the case of lysine is indicated by the single point shown for 22-hour and 44-hour growth.—The dotted lines indicate the character of the optical density-versus-time growth curves.

If only the 22-hour values are considered the responses are, over the respective ranges shown, almost directly proportional to the amino acid quantities, with a tendency toward concavity of the plotted curve, which in all cases except methionine corresponds to a difference of less than 5 per cent between relative growth responses at the 600 and 300 AOD levels. However, depending on the particular limiting amino acid both increases and decreases in optical density are evident subsequent to the initial 22-hour period. While in the cases of threonine and leucine substantial increases occur, growth remains stationary in the case of valine and shows a marked decline in the case of histidine and, very dramatically, in the case of lysine. The arrangement of the amino acids in Fig. 1 is thus in an approximate order of increasing degree of completion and decreasing stability of the initial growth. The nature of these reproducible characteristics remains to be explored. A study of the lytic phenomenon in the extreme case of lysine has already been reported (5).

Stokes and Gunness (3) have demonstrated a positive correlation between the quantities of different amino acids required for maximum growth and their relative amounts in the bacterial substance. As the present data permit an approximate evaluation of the absolute amounts of bacterial substance resulting from the utilization of definite quantities of different limiting amino acids they invite a further consideration of the relation pointed out by Stokes.

According to our determinations (6) an AOD of 600 corresponds to approximately 2000 micrograms of dry bacterial substance. The numerals shown in Fig. 1 give the microgram quantities of the respective amino acids required for its biosynthesis during 22 hours, and the data of Stokes and Gunness (3) give a measure for the amounts of those amino acids present in the bacterial substance. Comparison of the corresponding quantities for each of seven amino acids yields the ratios shown in column (d) of Table I. It is noteworthy that in all cases this ratio is of the order of magnitude of unity and in no case exceeds it. The possible significance of the apparent deficits of 20 to 30 per cent in this method of balancing cannot be evaluated without further detailed investigation. However, the cited work of Stokes (3), as well as the discovery of free amino acids in bacteria by Gale (1), indicate the probability of some variation in the amino acid content of the organisms, depending on culture conditions; and

of lysine is indicated by the single point shown for 22-hour and 44-hour growth.—The dotted lines indicate the character of the optical density-versus-time growth curves.

TABLE I
RELATIONS BETWEEN AMINO ACID CONTENT OF MEDIUM AND BACTERIAL CELLS

	(a) Amount found in dried cells (3)	(b) Approxim. amount occurring in 2000 γ bact. substance	(c) Amount required to produce AOD 600 (approxim. 2000 γ bact. substance) in 22 hrs. (Fig. 1)	(d) Ratio (c) : (b)
	per cent	γ	γ	
Leucine	4.5	90	76	0.85
Isoleucine	4.1	82	63	0.77
Valine	3.9	78	56	0.71
Arginine	3.0	60	44	0.73
Threonine	3.1	62	42	0.68
Methionine	1.5	30	29	0.97
Histidine	1.05	21	16	0.78

even in different batches grown under identical conditions, Stokes encountered differences of 10 to 20 per cent in their amino acid values, presumably because of the limited accuracy of the bioassay method.

Nevertheless, the fact that contrary to the relations between nutritional requirement and tissue retention of amino acids in higher animals, the bacterial ratio between amino acid consumption and amino acid retention is near unity, seems to justify the hypothesis that in the organism studied the indispensable amino acids are used only as actual or potential protein building blocks and serve neither purposes of energy metabolism nor as precursors of other amino acids. This hypothesis and the underlying evidence refer to conditions in which presumably all of the dispensable amino acids which normally occur in the bacterial organism are abundantly present in the medium. Accordingly, it becomes necessary to expect that omission from the medium of a dispensable amino acid may cause an increase in the requirement for one or several indispensable amino acids, which may thus be identified as precursors of the nutritionally dispensable tissue component. If no such increased requirements were found one would have to assume that the metabolism of the dispensable amino acids is entirely independent of the indispensable ones. These alternatives will be studied.

SUMMARY

In experiments with eight of the "essential" amino acids the initial amount of bacterial growth (S. faecalis 9790) induced in a phosphate-

buffered synthetic medium was found to be almost linearly proportional to the quantity of the limiting amino acid.

Quantitative considerations suggested the hypothesis that the amino acids indispensable for the growth of the bacterium are used only as protein building blocks, serving neither purposes of energy metabolism, nor as precursors of other amino acids when the latter are available preformed.

On continued incubation the shape of the response curve was found to change in the case of each limiting amino acid in a different, individual and reproducible fashion, as the result, apparently, of either additional growth, or lysis, or a stationary condition.

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THE EFFECT OF AQUEOUS SPLEEN EXTRACT ON GROWTH OF TUMOR CELLS IN MICE.

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The inhibitory effect of spleen extract on human tumors has been claimed for many years by clinicians, who based its use on the supposed ability of the spleen as an organ to suppress metastatic infiltration of tumor tissue from other sites and also on the low incidence of primary tumors in the spleen. The most recent conclusion in this connection is that of Woglom, who has interested himself in this problem for nearly thirty years (e.g. 11, 12). In a recent review (13) he declares that the hypothesis that the spleen is antagonistic to malignant growth is "immortal but mistaken."

Whether or not the spleen is able to suppress malignant growth within its own tissues, an inhibitory effect of extract of calf spleen on growth of certain mouse tumors was demonstrated by the work of Lewisohn (3, 4). More recently, Amersbach and his associates (1) reported complete regression of basal cell epithelioma in patients treated with extracts of human spleen and liver. The latter article contains an extensive review of pertinent literature. Another recent paper by Jones (2) reports that when tumor tissue was grown by him *in vitro* along with spleen tissue there was no suppression of tumor tissue growth nor was there suppression of "takes" on subsequent implantation of the tumor into mice.

As was reported in a preliminary way (10) the present authors were led to investigate further the effect of spleen extract on mouse tumor cells because of the clinical experience of the medical member of the group over a period of years that patients with malignant disease showed clinical improvement and prolongation of survival

time when aqueous extract of calf's spleen was injected, either alone or in combination with x-ray or surgery (9). A report of these clinical findings is being prepared for publication elsewhere.

The part of our investigations reported here concerns only the changes produced in mouse tumor cells and in the cells of the normal tissues of tumor-bearing mice injected with the Watson aqueous spleen extract.

Material and Methods

Most of our work was conducted on transplantable sarcoma 37 growing in albino mice of Carworth Farms stock reared in the Institute for Cancer Research in Philadelphia. This tumor has been used in our laboratory and also at the National Cancer Institute as the subject of extensive chemotherapeutic studies and its cellular conditions are well known. Sarcoma 180 implanted in the same stock of mice was also studied comparatively.

Tumors were injected by the trocar method and allowed to grow to a diameter of about 10 mm. (6-7 days), whereupon spleen extract was injected into the host mice, either by the subcutaneous or the intraperitoneal route.

In the case of primary tumors we used "A" strain mice bearing obviously growing methylcholanthrene-induced sarcomas of a volume at least 0.5 cu. cm. Caliper measurements were made at 3-day intervals.

Extracts were prepared in three concentrations, roughly approximated, and designated as "low," "medium," and "high," yielding a solid content of 0.5 gm./10 cc., 1.0 gm./10cc. and 1.50 gm./10 cc. respectively. The extract was tested for sterility at the close of the extraction process. Care was taken to keep it cold at all times, since it was found that it deteriorates rapidly at room temperatures. The preparations used in the Philadelphia laboratory were transported from Kitchener in constant contact with ice and were tested again for sterility[1] before injection. Extract thus preserved has a clear bright eosin-red color and a metallic sheen, whereas extract that is beginning to undergo deterioration takes on an increasingly muddy and brownish color. In our experience the extract is useless in effecting tumor cell destruction after such changes set in.

[1]The authors wish to thank the McNeil laboratories for making the sterility tests and bottling the extract.

Because of a tendency of sarcoma 37 of the stock now being maintained in the Philadelphia laboratory to undergo some spontaneous regression (at the present writing less than 4%) after the tumor has been implanted for about 18 days, most of our experiments were terminated before the tumors reached that age to avoid misleading evaluations. The tumors were then prepared for cytological study either by the acetic orcein smear method or by routine paraffin techniques.

Normal tissues of treated mice were studied in paraffin sections stained with hemalum and eosin, or in dry tissue imprints and smears stained with Giemsa and with May-Grünwald.

In a preliminary experiment, 4 groups of 20 animals (10 males and 10 females) each, bearing well-established 6-day sarcoma 37 implants evenly distributed as to size, were injected with 0.25cc. three times daily (total 0.75cc.) by the subcutaneous route. Because of preliminary experiments carried on at Kitchener, it was at first thought that heavy-bodied mice could tolerate larger amounts of the extract than could small bodied ones. The only heavy-bodied animals available to us were Swiss mice, all over 6 months of age, from the main colony of the Institute. The results of the experiment are indicated in Table I. Casualties were high in the group injected with high concentration, and the majority of the animals died after the total of the doses surpassed 2 cc. On low concentration the animals were not in good condition at the end of 3 days, and the tumors, with one or two exceptions, were growing. Of the 16 survivors of the medium concentration group, a marked superficial flattening of the tumors and inhibition of their growth was observed beginning at 3 days. At the end of 10 days, at which time some of the controls were already dead, the experiment was terminated. Tumors of mice injected with high concentration were macroscopically shrunken and hemorrhagic. In the group that had received medium concentrations, the tumors were found to be in every case smaller than those of the controls. Since this was only a trial experiment, no slides were preserved from this series.

In an attempt to improve host survival and tumor necrotization in "high" and "low" concentrations, respectively, 20 female mice bearing 6-day tumors were next injected. These mice were Carworth Farms stock, weighing, on the average, 20 grams, since it had been

TABLE I

SUBCUTANEOUS INJECTION OF SPLEEN EXTRACT

1. Heavy-bodied mice (over 6 months of age) bearing 6-day implants of sarcoma 37

GROUP	No. of mice	Conc. of extract	Amt. injected	Times daily	Total daily dose	No. of days treated	Total dose	Casualties No.	Casualties Day of treat.	No. of mice surviving	Condition of tumors
I	10 ♂ 10 ♀	"High"	0.25 cc.	3	0.75 cc.	10	7.5 cc.	16	1st–3rd	4	Shrunken and hemorrhagic
II	10 ♂ 10 ♀	"Medium"	0.50 cc.	3	1.50 cc.	"	15.0 cc.	4	1st–3rd	16	Decreased in volume
III	10 ♂ 10 ♀	"Low"	1.0 cc.	3	3.0 cc.	"	30.0 cc.	6	1st–3rd	14	Smaller than controls, but growing
IV	10 ♂ 10 ♀	Controls	—	—	—	—	—	9	5th–10th	11	Growing rapidly

2. Young mice (8 weeks old—average weight 20 grams) bearing 6-day implants

I	20 ♀	"High"	0.5 cc.	3	1.50 cc.	2	2.5 cc.	4	2nd	16	— Tumors inhibited
						3	—	13	2nd–3rd	3	— Tumors hemorrhagic
						4	5–6 cc.	2	3rd–4th	1	— Tumors regressing
						5	6–7 cc.	1	5th	0	— Tumor static
II	20 ♀	"Low"	0.75–1 cc.	3	2.25–3 cc.	1	2.75 cc.	1	2nd	19	— Tumor of dead mouse shrunken
						5	15.0 cc.	1	5th	18	— Tumor resorbed
						10	30.0 cc.	—	—	18	— 5 tumors completely necrotic; 13 inhibited
III	20 ♀	Controls	0.5 cc. saline	3	1.50 cc.	13	20.0 cc. saline	1	10th	15	Growing, some central necrosis

observed that older mice, such as we used in the first experiment, did not tolerate treatment as well as did young ones. Each animal was injected 3 times daily unless it appeared to be in poor condition, as was sometimes the case in the first few days of spleen injection. Animals that survived 2 cc. of the extract apparently developed a tolerance and usually could be treated 3 times daily thereafter. The individual doses were the same as those used in the preceding experiment and the results appear in the second part of Table I.

The appearance of the animals treated subcutaneously was not good. Multiple injections left many lacerations in the skin, from which extract sometimes oozed, so that it was impossible to determine exactly how much was retained. The animals appeared unwilling to accept food and became extremely gaunt. Post-mortem examinations of animals that died during treatment revealed that the extract frequently coagulated in pockets beneath the skin and was not readily absorbed. For these reasons, it was decided to attempt intraperitoneal injection, reducing the dose, if necessary, to fall within the limits of tolerance. Experiments were accordingly made on 6-week old normal mice (males and females) without tumors. It was found that non-tumor-bearing mice could tolerate 4 injections (0.1 cc. each) daily of high concentration extract and three or four injections of medium concentration extract totalling 1 cc. daily, six hours apart, no injections being made during the night. The low concentration was abandoned as being too feeble a necrotizing agent in the doses that could be tolerated by our mice.

To test the ability of tumor-bearing animals to tolerate intraperitoneal injections of this magnitude, 20 female mice bearing 7-day tumors were injected 3 times daily with medium concentration extract in doses of 0.35 cc., 0.30 cc., and 0.35 cc. respectively. During the 12 days of experimentation there were no casualties either in the treated animals or in the controls. Two tumors were completely resorbed, and microscopic examination revealed that the remaining tumors were either non-viable or showed varying amounts of tumor cell necrotization greatly in excess of that exhibited by the controls (Table II).

In another experiment with fewer animals (see second part of Table II) we repeated this experiment, raising the number of injections to 4 per day at 6-hour intervals around the clock in both

TABLE II

INTRAPERITONEAL INJECTION

1. *Female Swiss mice bearing sarcoma 37 implants, treated 3 times daily with spleen extract (total 1 cc. daily).*

Days under treatment	No. of animals	Total dose per animal	Concentration	Casualties	No. of animals surviving	Condition of tumors	Cytological findings
1	20 ♀♀	1.0 cc.	Medium	0	20		
2		2.0 cc.	"	1	19	Hemorrhagic	
3		3.0 cc.	"	6 (after 2 cc.)	13	Necrotic	Pycnotic nuclei; enucleated cells
4		4.0 cc.	"	0	13		
5		5.0 cc.	"	0	13		
6		6.0 cc.	"	0	13	1 tumor resorbed	
7		7.0 cc.	"	1 dead (6.2 cc.) 1 dead (6.5 cc.)	11	Shrunken	No viable cells
8		8.0 cc.	"	0	11		
9		9.0 cc.	"	0	11	1 resorbed	
10		10.0 cc.	"	0	11		
11		11.0 cc.	"	0	11		
12		Treatment discontinued		—	—		
				9	11	3 growing 2 partly sloughed; 4 static 2 resorbed	3 tumors dividing; 3 fragmented and vacuolated nuclei; 3 resting cells and fragments
1	14 ♂♂	0.70 cc.	Medium	0	14		
2		1.40 cc.	"	0	14		
3		2.40 cc.	"	0	14		
4		3.65 cc.	"	0	14		
5		4.65 cc.	"	1	13		
6		5.65 cc.	"	1	12		
7		6.65 cc.	"	0	12		
8		7.65 cc.	"	2	10		
9		8.95 cc.	"	1	9		
10		9.95 cc.	"	0	9	1 tumor resorbed	
11		10.95 cc.	"	0	9		
12	No injections.						Partial fragmentation; vacuolization; polyploid mitoses.
13		11.45 cc.	"	1	8	3 tumors resorbed	
14		12.45 cc.	"	0	8		
15	Experiment terminated:			—	—	—	
				6	8	4 resorbed; 4 growing	
Controls	14 ♀♀ 14 ♂♂	———		0	14	tumors growing	

TABLE II (Continued)

INTRAPERITONEAL INJECTION

2. *Male Swiss mice bearing 7-day sarcoma 37 implants injected 4 times daily*

Days under treatment	No. of animals	Total dose per animal	Concentration	Casualties	No. of animals surviving	Condition of tumors	Cytological findings
6	8 ♂♂	2.4 cc.	High	1 dead	7		Coagulated cells (post-mortem change?)
16	Experiment terminated	6.4 cc.	"	—	7	Tumors growing	
3	8 ♂♂	3.0 cc.	Medium	1 dead	7	Tumor shrunken	Pycnotic cells
11–13		11–13 cc.	"	0	7	5 tumors completely resorbed	
16		16.0 cc.	"	0	7	2 remaining tumors resorbed	No living tissue remaining for microscopic study
Controls	8 ♂♂	——	——	—	8	1 tumor partly sloughed; 2 ulcerated	Spot necrosis and dividing cells; healthy resting nuclei.

medium and high concentration. Within twelve days *all* of the animals that had received medium concentration extract (total 13 cc.) had resorbed their tumors. Apparently increased frequency of injection improved the efficacy of the extract even when the daily quota was not increased. The amount of high concentration extract (total 4.0 cc.), however, was not sufficient to cause resorption and most of the tumors in this group showed some mitotic figures.

For the rest of our studies, therefore, we used only medium concentration, introduced by the intraperitoneal route, establishing tolerance gradually by injecting only once or twice daily until the animals had received a total of 2 cc. each, and thereafter 3 or 4 times daily to the amount of at least 1 cc. per day per animal. This procedure was found to yield the optimum result both as to survival and tumor necrotization.

Several hundred treated tumors have since been studied microscopically.

Microscopic observations

a. Transplanted tumors

As noted above, the optimum concentration of the Watson extract to secure tumor necrotization without loss of life in mouse hosts was 1 cc. daily of the "medium" concentration injected in 3 or 4 individual doses. Tumors (S-37) thus treated were inhibited in growth beginning on the third day. For microscopic study we therefore preserved tumor tissue from the fourth day of treatment onward and continued to dissect tumors daily until no more growing tissue could be detected macroscopically in the respective tumors. With the medium concentration (total 2 cc. of extract) in tumors preserved on the third day a picture of almost complete degeneration (fig. 5) was usually encountered. The tumor nuclei were fragmented into irregular masses or were granular and filled with vacuoles. Underlying these fragments, individual cell bodies remained intact, so far as the cytosome was concerned, but many of them were entirely enucleate and obviously moribund. Others had shadowy residues of nuclear material, which was either non-orcein staining, or stained a muddy gray. At times the cytoplasm was also filled with small vacuoles, depending on the degree of degeneration. Undamaged cells were still in division. On the fifth day (total 5 cc.)

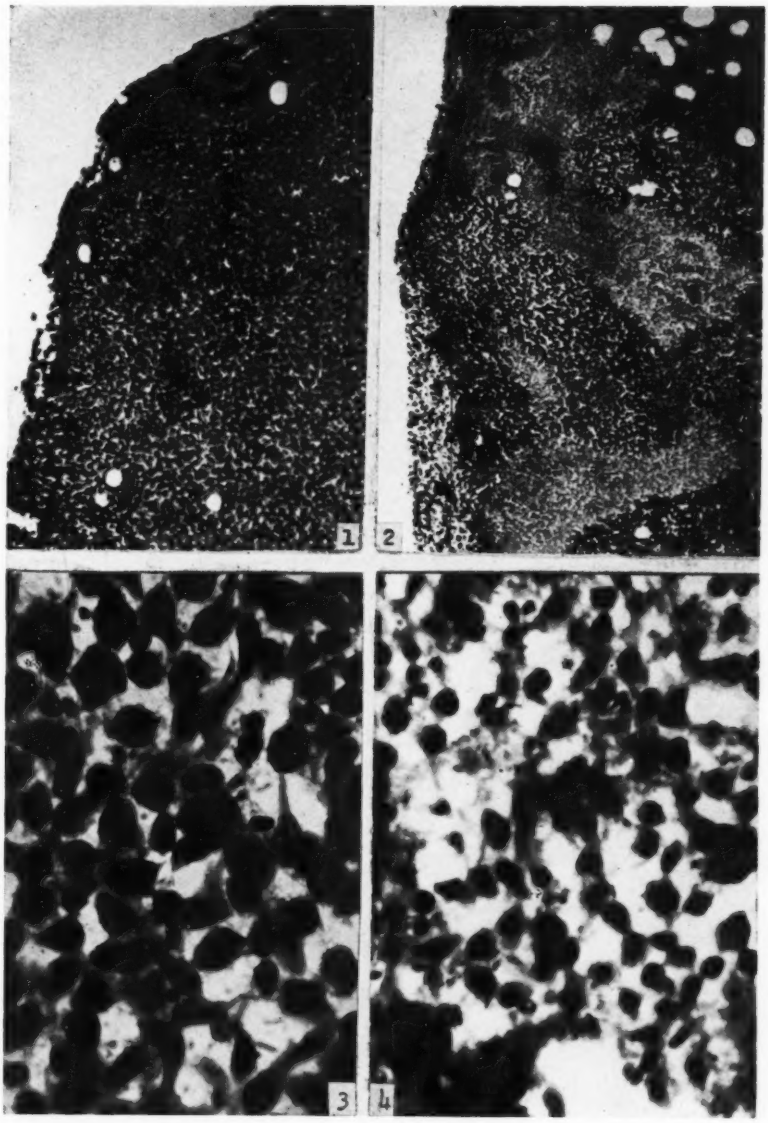

PLATE I

Figure 1—Untreated sarcoma 37, paraffin section stained with hemalum and eosin. Magnification 100 X.

Figure 2—Sarcoma 37 treated with spleen extract for 7 days (7 cc. of medium concentration) showing necrotic peripheral indentations. Magnification 100 X.

Figure 3—Untreated sarcoma 37, acetic orcein smear. Magnification 440 X.

Figure 4—Sarcoma 37, acetic orcein smear of tumor treated with 1.50 cc. of high concentration spleen extract injected intraperitoneally.

inroads of necrotic tissue around the periphery (fig. 2) were observable in tumors prepared by the paraffin method. The peripheral necrosis proceeded in a roughly scalloped pattern, that is, irregular indentations of dead tissue appeared all around the outer edge, following capillary pathways, and these increased in size from day to day, with viable tissue, still in mitosis, shrinking towards the center. Whatever the mechanism whereby necrotization is effected, spleen extract evidently is not a mitotic poison, for no matter how small the residue of living tissue, cells in mitosis could still be found.

The effect of subcutaneous injections of the medium concentration was also studied. Cell degeneration was evident under this treatment, but the amount of spleen extract required to effect definite damage was much greater than was required when the extract was injected intraperitoneally, and the number of tumors that responded, fewer. On the sixth day (total 6 cc. of spleen extract) greatly enlarged resting nuclei were observed. Dividing cells were present and there were many polyploid figures. In some cells the chromosomes appeared unusually thick. Other aberrant mitotic figures were observable and some telophases showed chromosomes at one pole which were thick, heavily stained and doubled in number, while the chromosomes at the other pole of the spindle were of normal number and thickness. Not until animals had received 8 cc. of extract subcutaneously could definite nuclear damage be detected (fig. 6) but the pattern of degeneration was similar to that obtained with intraperitoneal injection of smaller amounts.

Observations were made also on tumors from animals surviving injection of the high concentration. Following a single injection of 0.5 cc., an animal dead at 5 hours post-injection showed that reaction had taken place, and many nuclei were markedly hypertrophic. The number of nucleoli was not increased, indicating that enlargement of nuclei was probably due to uptake of fluid, rather than to increase in chromosome number. After a mouse host had received a total of 2 cc. of high concentration extract, considerable haziness of staining and the beginning of nuclear degeneration were encountered. Many nuclei disintegrated completely, leaving behind small groups of granules and vacuoles. Dividing cells were still present and undamaged, although many of them were polyploid. Large numbers of lymphocytes were found throughout the tumor.

When the high concentration was given intraperitoneally (3 doses of 0.1 cc. daily) animals still alive at 75 hours showed definitely degenerative tumors (fig. 4). The nuclei, sometimes shrunken and bean shaped, were stained hazily with orcein and were vacuolate. Individual chromatin threads could not be made out distinctly, and frequently the entire center of the nucleus was one large vacuole. Others were stained only at the rim.

With low concentration extract much larger doses were needed to cause nuclear destruction. Figure 7 shows the effect obtained in sarcoma 37 following the injection of 12 cc. of low concentration extract intraperitoneally. The picture is one of almost complete cell destruction. The beginning of such destructive changes, consisting of a partial coagulation of the chromosomes, and haziness of staining in orcein smears, was first noted on the third day (total 6 cc.). Resting nuclei at that time were swollen and deeply stained.

The results obtained with sarcoma 180 were similar except that a greater amount of spontaneous necrosis in control tumors at 7-10 days rendered the evaluation of spleen extract effects difficult.

The most rapid and notable necrotization was obtained with the high concentration extract injected intraperitoneally; but casualties were also high. Low concentration, even when intraperitoneally injected, was slower in producing results, and growth inhibition was less marked. Apparently the effects obtained are quantitative.

A small amount of high concentration extract was lyophilized and the dried powder redissolved in sterile salt solution in an amount equal to the original volume of fluid. This preparation was injected intraperitoneally into mice with 7 day tumors and the tumors were studied microscopically after 3 days to determine whether or not freezing and drying had destroyed the potency of the extract. Fig. 8 shows the appearance of such a tumor 75 hours after initial injection (1.5 cc.). Degeneration similar to that obtained with non-lyophilized extract is present, and the damaged nuclei, stained in orcein, appear as grey spheres with heavily stained rims. No animals died under treatment.

b. Primary tumors

When "A" strain mice bearing small (0.5-0.8 cu. mm. in volume) methylcholanthrene-induced tumors were injected intraperitoneally

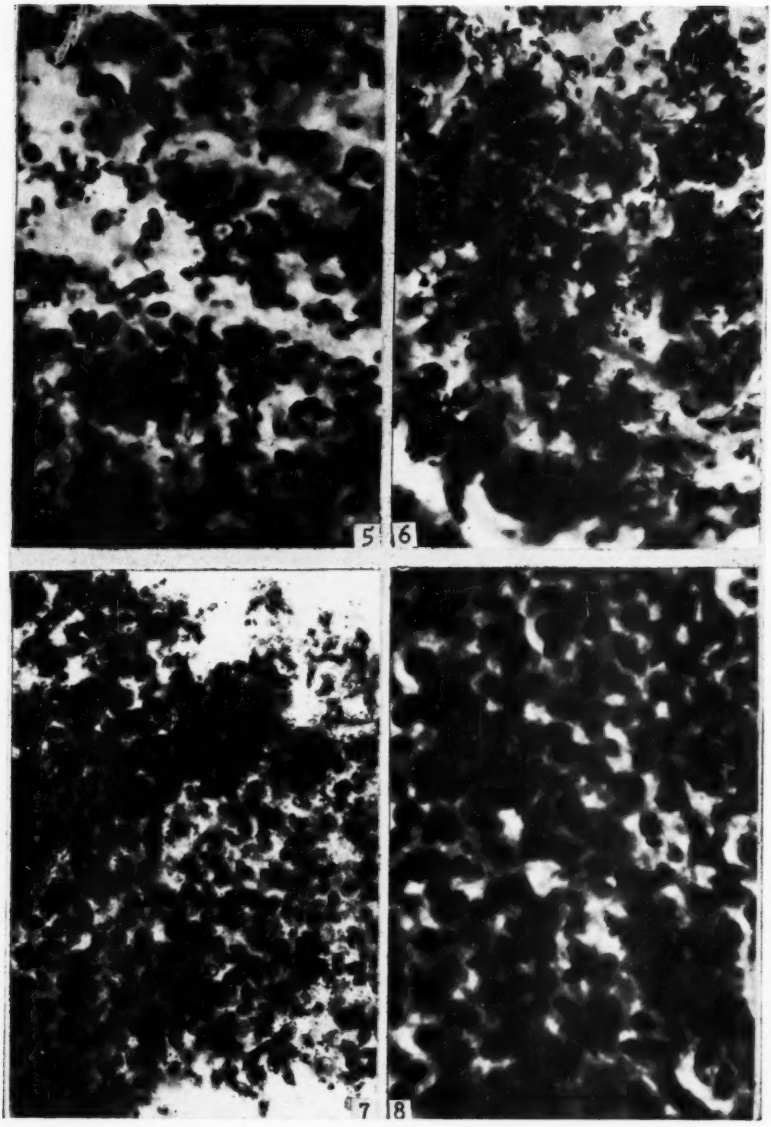

PLATE II

Figure 5—Sarcoma 37, acetic orcein smear of tumor after injection of 2.0 cc. of spleen extract, medium concentration. Magnification 440 X.

Figure 6—Sarcoma 37, acetic orcein smear of tumor treated with 8.00 cc. of spleen extract, medium concentration, subcutaneously. Magnification 440 X.

Figure 7—Sarcoma 37, acetic orcein smear of tumor after injection of 12.00 cc. of spleen extract, low concentration, intraperitoneally. Magnification 440 X.

Figure 8—Sarcoma 37, acetic orcein smear of tumor treated with 3.00 cc. of lyophilized extract (derived from high concentration). Magnification 440 X.

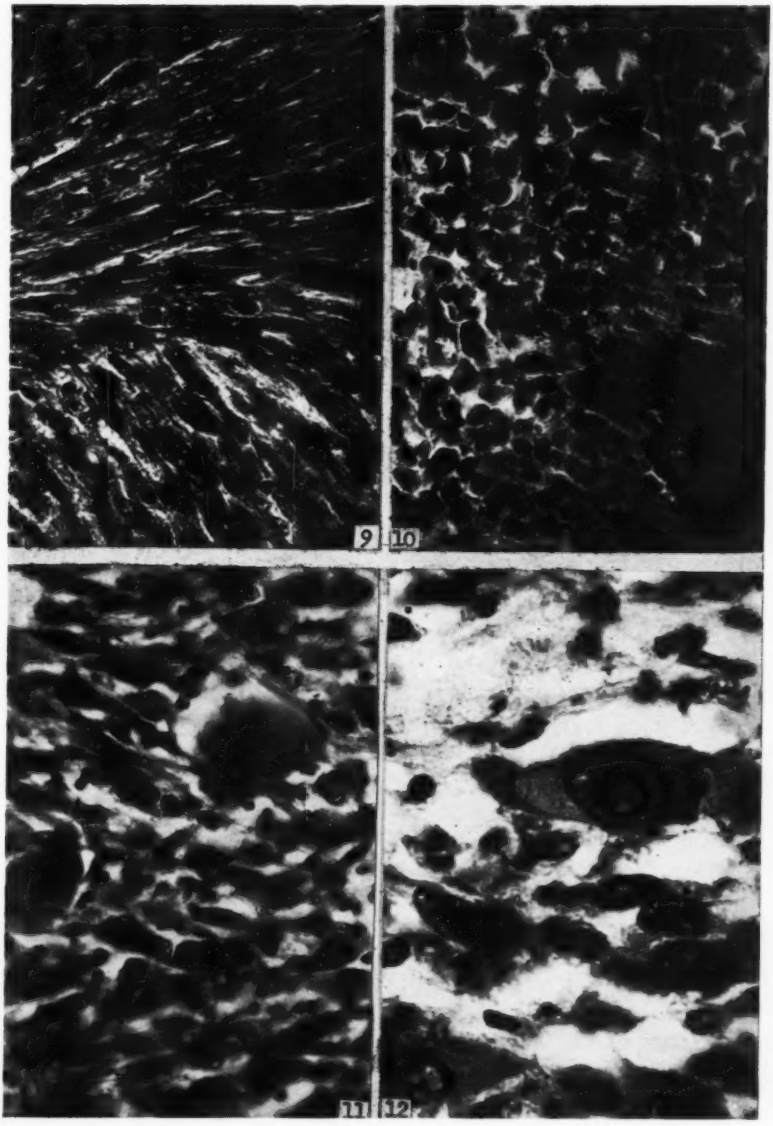

PLATE III

Figure 9—Untreated methylcholanthrene-induced sarcoma, paraffin section stained in hemalum and eosin. Magnification 440 X.

Figure 10—Methylcholanthrene-induced sarcoma, paraffin section stained with hemalum and eosin, treated with 7.00 cc. of spleen extract, medium concentration, injected intraperitoneally. Magnification 440 X.

Figure 11—Methylcholanthrene-induced tumor treated with 3.00 cc. of spleen extract, medium concentration, intraperitoneally, showing invasion of tissue by giant cell. Magnification 440 X.

Figure 12—The same as No. 11, at higher magnification, showing photocytic nature of giant cells. Magnification 900 X.

with medium concentration extract 3 times daily (total 0.75 cc.) inhibition of tumor growth began on the fourth day. In about a week some of the tumors were definitely smaller. Resorption of the tumors was slow and they sometimes remained static for several weeks, eventually regressing completely. Larger tumors were only temporarily inhibited at this dose level. Mice whose tumors were completely resorbed and which have not been treated since February, 1948, have shown no sign of recurrence to date (December, 1948). Spontaneous resorption of these chemically induced tumors, once they have begun to grow, has not been observed in this laboratory.

Tumors were preserved for microscopic study as soon as definite shrinkage in size was noted (one week after beginning of treatment). The appearance of such tumors is shown in figure 10. Instead of long, slender nuclei and spindle shaped cells joined end to end in a chain-like pattern, found in the controls (fig. 9), these degenerating nuclei have become pycnotic and shrunken and the individual cells bodies are disjoined and coagulated. Earlier (3 to 4 days after beginning of treatment) the tumor tissue, though mostly undamaged, was invaded by giant cells (fig. 11, 12). During later stages of tumor degeneration these cells became phagocytic, engulfing not only debris but whole tumor cells as well.

c. Normal tissues.

A study was made of the normal tissues of the animals whose tumors have been described above and also of post-mortem specimens from animals that died under treatment. No tissue damage referable to spleen treatment could be detected. In fact, the picture is rather one of repair, or at least of stimulation of mitosis, particularly in the blood forming organs. In the spleen itself, as Lewisohn (4) has shown, there was marked increase in the number of macrophages. Beginning with the third day after intraperitoneal injection of medium concentration extract, increase in mitotic activity was observed in the fixed tissues of the hemopoietic system, notably in the lymph nodes and the thymus (particularly the medulla of the thymus).

Because of this obvious stimulation of the blood forming organs, a special study was made of the hemapoietic system. Smears of the

circulating blood were made before and after treatment, the blood being drawn from the tail of the mouse. Mice injected with medium concentration extract were killed at daily intervals and smears of the lymph nodes, spleen, thymus, bone marrow, liver and of the tumor were made by the dry tissue imprint method. The imprints were stained with Giemsa and May-Grünwald in each instance. The remainder of the solid organs was preserved in paraffin and studied in sections stained with hemalum and eosin.

On the fourth day of treatment with extract of medium concentration injected intraperitoneally, lymph tissue showed numerous dividing lymphoblasts; the spleen also was in division, with histiocytes in prophase. No change was noted in the liver up to the fourth day.

On the fifth day the wave of mitosis in the lymph nodes was apparently ebbing, but the thymus continued in active division, as did the spleen, and mitotic figures began to appear in the liver. On the sixth day, when many of the tumors were necrotic and largely resorbed, division in the lymph tissue had subsided. Both the spleen and the liver contained unusual numbers of macrophages. In the circulating blood the percentage of lymphocytes rose steadily from the second to the fourth day after the beginning of injection, and at that time many lymphocytes began to appear at the site of the tumor. Thereafter the percentage of lymphocytes in the circulating blood declined and reached the level of the controls on the sixth or seventh day.

Increased mitotic activity was usually encountered in the cortex of the adrenal gland on the eighth or ninth day of injection.

Discussion

The most interesting finding that emerges from these studies is that the amount of tumor cell destruction is proportional to the amount of extract that can be injected without causing death of the tumor host. Apparently it is necessary to employ as much extract as can be tolerated by the mouse, and the best way of accomplishing this is to divide the daily quota into three or four injections. This is particularly important in the beginning of the experiment in order to overcome rapid proliferation. Apparently this was the case also with the extract used by Lewisohn, who reported 60% regression of transplanted mouse tumors under daily intravenous injections of

his concentrated extract. No effect was obtained by him in spontaneous breast carcinoma of the mouse under the same procedures. It was not until he had injected an extract of the "healed" spleens of treated mice, whose tumors had regressed completely under spleen extract therapy, that he was able to obtain regression of spontaneous tumors. He believed that the enlarged "healed" spleens had concentrated a greater amount of the agent responsible for tumor inhibition. In view of the finding of Amersbach *et al* that human epitheliomas disappear under treatment with human extracts (1), one wonders whether the improved results obtained by Lewisohn, when tumor bearing mice were injected with extract of the "healed" spleens, were not, in part, due to the fact that he was using a homologous extract.

With the extract employed by us, and possibly because of the multiple daily intraperitoneal injections, it was not necessary to resort to intravenous injection in order to secure tumor cell necrotization in transplanted or methylcholanthrene-induced tumors.

In Lewisohn's "healed" mice he found the spleen to be much enlarged. This he attributed to increase in the number of macrophages. No great enlargement of the spleen was found in our animals during the 7 to 10 days required to effect tumor necrotization.

In our experiments, the tissues of the body most affected were those concerned with the proliferation of blood cells, and particularly those tissues which produced cells associated with antibody formation. Over twenty years ago Murphy and Sturm (7) and Murphy and Nakaharo (8), found that stimulation of lymphoid elements result in the establishment of a high degree of immunity to certain transplantable tumors in mice. Mice injected by them with defibrinated blood showed in the germinal centers of the lymphoid organs a marked increase in the numbers of mitotic figures. The increase became evident forty-eight hours after injection and reached its climax about the fifth day. These immunized animals showed a high resistance to transplanted tumors. Murphy and Morton (6) worked also with mice bearing spontaneous tumors, which, when exposed to small doses of X-ray sufficient to stimulate lymphocyte production, showed resistance to replants of fragments of their own tumors. Possibly spleen extract works in somewhat the same way as defibrinated blood to increase the activity of cells concerned with the formation of immune bodies.

In a recent paper Macfarlane and collaborators (5) reported vacuolization of nuclei, disappearance of nucleoli, reduction in size of heterochromatic chromosome segments and inhibition of mitosis when cells of certain spontaneous tumors were stored in concentrated aqueous spleen extract for varying lengths of time at 5°C. They conclude that spleen extract under such conditions has a direct effect upon tumor cells. Following injection of spleen extract we noted also vacuolization of nuclei and enucleation (p. 37) but the conditions under which Macfarlane's experiments were performed are so different from those under which we worked that we probably cannot justifiably compare results. Jones' (2) report that no cell damage is effected when tumor tissue is grown *in vitro* with spleen, skeletal muscle and kidney tissue from non-tumorous animals probably has little bearing on Macfarlane's findings either. If the suppressive effect does arise through stimulation of the reticulo-endothelial system it would not be reasonable to expect that we should have direct inhibition of tumor cell multiplication when spleen tissue and tumor tissue are grown together *in vitro* in the absence of the reticulo-endothelial elements. Since no cell damage could be detected in normal tissues of treated mice, no matter what the dosage, and since mitotic figures persisted as long as tumors were viable in any part, we are inclined to attribute death of tumor cells under spleen extract therapy, not to direct destructive action on cells, but rather to some indirect process.

Because of the apparent relationship existing between the amount of spleen extract injected and the amount of tumor cell necrotization, some means must be found for standardizing the extract. Fractionation methods should be employed, but in such a complex substance this will be a major task. Because of the response of sarcoma 37 to lyophilized spleen (fig. 8) it is our hope that in the near future lyophilization of the extract can be carried out on a large scale, so that sufficient amounts will be available for adequate testing of a large series of dilutions, with many animals and by different injection routes.

Summary

1. The injection of aqueous extract of calves' spleen produced, in transplanted mouse sarcoma 37 and methylcholanthrene-induced primary sarcoma in "A" strain mice, degeneration of tumor cells,

provided an adequate concentration was employed. The best results were obtained with a medium concentration (1.5 gm./10cc.) injected four times daily.

2. The cell changes observed consist in vacuolization of the cytoplasm, nuclear pycnosis or granulation, and coagulation of the chromatin. Undamaged cells became greatly enlarged and polyploidy in these cells was unusually frequent. Cells which were not destroyed continued to divide, with a notable increase in the number of multipolar spindles.

3. The normal tissue cells were unaffected by spleen extract injection except that there was a stimulation of mitosis in all of the blood forming organs, particularly the lymphoid tissues. The spleen was filled with macrophages and dividing hemoblasts, and the medulla of the thymus showed unusual mitotic activity. Lymphocytes and macrophages were found in the tumor tissue in large numbers, both in transplanted and chemically induced tumors.

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Growth, 1949, **13**, 45-70.

ONSET OF OSSIFICATION IN THE EPIPHYSES AND SHORT BONES OF THE EXTREMITIES IN CHIMPANZEE*

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INTRODUCTION

As a technique for the study of growth, the analysis of skeletal development by means of serial roentgenograms offers unique advantages. In contrast to anthropometric measurements, it provides growth indices for one specific tissue rather than a composite measure of several body components and permits exact measurements which may be repeated and checked at any later time. In contrast to the study of cadavers, it makes possible longitudinal studies of the living organism, with the postnatal growth period of the individual divided into whatever intervals may be desired. Finally, the growth of bone is in general less influenced by minor and transient variations in environmental conditions, use, and health than is that of other tissues.

The indices of skeletal development derivable from roentgenograms may be divided into five main classes: (a) changes in size—especially length and cross-section—of individual bones and in the ratios of such dimensions between various bones; (b) changes in contour and shape, especially of the carpals and tarsals; (c) changes in density and other fine structural characteristics of bone; (d) time and order of appearance of various centers which first begin to ossify postnatally: most of the epiphyses and short bones of the extremities; (e) age at the time of fusion of the various epiphyses with their corresponding diaphyses. The present report deals entirely with

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indices of class (d): the ages at which various centers of the extremities first begin to ossify in chimpanzee. Such data are available for man and have been treated to provide norms for age and age-variability in appearance of specific centers, for rate of appearance of new centers, and for order of appearance (1, 2, 3, 4, 9, 12). These statistics have found application in the discovery of deviations produced by various genetic and environmental factors such as sex, disease, and nutritional status. Many further possible relationships, as for instance with length of gestation, individual heredity, dentition, weight and other measures of growth, have thus far received little attention.

For primates other than man, information on the sequential postnatal appearance of ossification centers is extremely meager (11). What there is, has been obtained almost entirely by the cross-sectional rather than the longitudinal method, and much of this information is only approximately correct, since ages were often estimated on the basis of inexact criteria such as weight and dentition. Reliable data on this aspect of growth for infrahuman primates, and particularly for chimpanzee, should serve two general functions: (a) Since these animals are being employed increasingly in various fields of biological research, such measures would be useful in providing another set of criteria for normality of the biological materials and for estimating the effects of experimental variables (e.g. hormonal deprivation or supplementation, special nutritional conditions, and so on). (b) The data would provide opportunity for making species comparisons (especially with man) in respect to absolute age, variability, order of appearance, and the correlations of these with other available developmental measures. The determination of such species differences and correspondences is, of course, basic to the identification of principles of evolutionary change and relationships within the order.

The primary aim of this paper is to present normative data on the age and order of onset of ossification in seventy centers of the left extremities in chimpanzee infants, raised from birth in a uniform nursery environment. Subsequent reports will deal with the variations produced by differing conditions (e.g., infants nursed by their own mothers, castration, brain operations), with other growth indices derived from roentgenograms and anthropometric measurements, and

with the inter-relationships among various physiological and behavioral measures from the same group of chimpanzees studied from birth to maturity.

Materials and Procedure

Subjects

The sixteen subjects of this study were born at the Yerkes Laboratories between July 9, 1939 and January 16, 1943. Genetic relationships among these animals, as well as the birth weight and gestation length of each, are presented in table 1. (Of the five other

TABLE 1
Showing Genetic Relationships Among the 16 Subjects, Gestation Length in Days, and Birth Weight in Kilograms

MOTHERS	FATHERS Jack	Bokar	Frank
Wendy	Jed (223 days) (1.86 kg.) Jenny (220 days) (1.65 kg.)	Web (204 days) (1.36 kg.)	
Soda	Spratt (228 days) (1.96 kg.)		Ken (232 days) (1.99 kg.) Scarf (231 days) (2.02 kg.)
Nana			Fanny (234 days) (1.83 kg.)
Josie	Jojo (222 days) (1.85 kg.)		
Fifi		Art (227 days) (1.99 kg.)	
Bula			Karla (236 days) (1.93 kg.)
Bimba			Banka (241 days) (1.84 kg.)
Alpha			Alf (235 days) (2.14 kg.) Flora (220 days) (1.94 kg.) Falla (227 days) (2.05 kg.)
Bentia	Jent (238 days) (1.72 kg.)		
Dita		Bard (207 days) (1.46 kg.)	

viable infants born here during this period two died, one was transferred to another laboratory, and two were raised by their mothers and therefore were not available for periodic examination.) Thirteen of the animals were separated from their mothers within 48 hours, the others 3, 8 and 14 days, respectively, after parturition. All were raised in the nursery cribs and cages under conditions of nutrition, handling and environment which were as uniform as individual idiosyncracies (e.g. of appetite) permitted (6). Illnesses, such as colds, sore throats, fungus skin infections, and gastrointestinal "upsets" occurred variably within the group, but these were usually of brief duration. As far as is known the only serious disturbances of health occurred in the form of amebic and bacillary dysenteries after the animals were over 30 months old. Generally accepted nutritional standards for human infants, including vitamins and mineral requirements, were met. The mean weights of our subjects at birth and at ages of one, two, three and four years were as follows: males, 1.83, 7.34, 10.76, 14.02 and 17.40 kilograms; females, 1.87, 7.24, 10.86, 14.79 and 18.45 kilograms, respectively. It may be noted in this connection that the rate of weight-gain is much greater, initially, for nursery-raised infants than it is for infants raised in the Laboratories by their mothers; weight at one year is roughly four times birth weight in the former, whereas it is only about 2.5 times the birth weight in the latter. This difference decreases as the nursery and mother-raised youngsters of both groups become increasingly independent in their feeding activities.

Procedure in making the exposures

The X-ray films used in this study were made with a 1/10 or 1/20 second exposure, the animal being positioned by the regular attendants. At the older ages, Nembutal anaesthesia was used with the less docile animals. Tube-film distance was constant at 41 inches. A table-top constructed of clear 1/4-inch plywood was used, this being less objectionable to the chimpanzees than the hard, smooth surface of the cassettes (par-speed screens were used) or of the standard, manufactured tables. Ten MaS were used for all exposures; the KVP varied from 32.2 (hand at birth) to 54.1 (trunk at 48 months).

Schedule of longitudinal records

The materials of this study are serial roentgenograms, of nine male and seven female chimpanzees, from which ages at onset of ossification in 70 centers were determined. Included in this count are all epiphyses and short bones of the left extremities with the following exceptions: lesser tubercle and lateral epicondyle of the humerus, olecrannon, calcaneus and talus. The latter two tarsal bones are well developed at birth in man as well as in chimpanzee. Sesamoids are not considered in this report.

Within the limitations set forth below, a set of roentgenograms was taken shortly after birth and at two-month intervals thereafter to the age of one year. From then until the animals were 4 years old, exposures were made every three months (i.e., at ages 12, 15, 18, . . . 45, 48 months). Omissions or gaps in this schema were caused by the following circumstances: 1) the program did not get under way until several months after some of the subjects were born; 2) one animal died (of an acute infectious disease) and two were transferred to other Laboratories before attaining the age of 4 years; 3) a very few scheduled sessions were missed or the films themselves were not of sufficiently good quality to permit an adequate assay. Table 2 shows, for each of the sixteen subjects, the ages at which complete and adequate sets of roentgenograms were obtained. In this table the animals are listed, within each sex-group, in the order of decreasing rate of ossification (see tables 4a and 4b below). A "set" included at least the following separate exposures:

1) Left hand, P-A (including lower parts of ulna and radius)
2) Left forearm, A-P (including wrist and distal humerus)
3) Trunk, A-P (including the shoulder and hip joints)
4) Left lower leg, A-P (including distal femur)
5) Left foot, A-P
6) Left foot, lateral

Up to the age of about two years the A-P (anterior-posterior, the anterior surface being nearest the tube) trunk picture included a lateral exposure of the knee joint; as the animals became larger, a separate exposure was made of the hip (A-P) and left knee (lateral).

In addition to a complete "set", thus defined and obtained at the intervals shown in table 2, anterior-posterior roentgenograms of the

TABLE 2

SHOWING AGES AT WHICH COMPLETE (X) AND PARTIAL (P) SETS OF ROENTGENOGRAMS WERE OBTAINED FROM EACH SUBJECT. LOWER LEG AND FOREARM PICTURES TAKEN AT MONTHLY INTERVALS NOT INCLUDED

Age in months	0	2	4	6	8	10	12	15	18	21	24	27	30	33	36	39	42	45	48
Scarf	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Jed	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Spratt	X	X	X	X	X	X	X												
Art								P	X	X	X	X	X	X	X	X	X	X	X
Ken						P		P	X	X	X	X	X	X	X	X	X	X	X
Alf							X	X	X	X	X	X	X	X	X	X	X	X	X
Jent	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X				
Bard					X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Web	X	X	X	X	X	X	X	X	X	X	X	X							
Falla	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X				
Flora	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Banka	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Jenny		P	P		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Karla	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X		X		X
Jojo	P	P		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Fanny	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X

forearm and lower leg only (Nos. 2 and 4, above) were made at each monthly interval not included in the table (i.e. at 1, 3, 5, . . . 13, 14, 16, 17 . . . months of age.) These pictures, since they included three of the principal joints, helped towards a precise determination of the centers located there.

The so-called birth pictures (0 months) were taken soon after separation of the infant from its mother, and never more than 48 hours after parturition. With a very few exceptions, subsequent pictures were taken not more than one day before or three days after the stated age in months.

Age-assessment of ossification centers

Centers clearly visible and of appreciable size on the birth pictures were recorded as "PN", indicating that ossification began prenatally. For this study, no attempt was made to differentiate order of appearance among PN centers; in some of our statistical calculations, below, an arbitrary value of minus one month has been assigned to all PN's. When the shadow of a center was just barely visible at the expected location, and if this shadow increased in size on subsequent pictures, it was recorded as having started ossification at the age (e.g. 4 months) of the first picture. If, on its first appearance, the center was already of considerable size, the assigned age was extrapolated backwards in accordance with its size and its more or less known rate of size-growth. Thus, a shadow definitely absent on the two-months picture but of considerable magnitude on the four-months picture would be assigned an onset-age in the range 2.5 to 3.5 months. In view of the fact that a full set of roentgenograms was taken every two or three months, the information furnished by these often being supplemented by the monthly forearm and lower leg pictures, this procedure seems not only justified, but more accurate than the lumping together, at a single age, of all centers which appeared since the previous picture was taken.

In view of the fact that the relative positions of certain centers at onset—especially of the carpals and tarsals—are not quite the same in chimpanzee as in man, it was sometimes necessary to trace the development of a short bone to an advanced age before being sure which bone we were actually dealing with. The longitudinal nature of the study, of course, contributed towards accurate identi-

fication. Some epiphyses and short bones (in some animals) began ossification from two or more centers, which later fused. Such multiple beginnings might be simultaneous or successive; in the latter case, the first one to appear determined the assigned onset-age.

Normative Chimpanzee Data

The basic data of this study are given in tables 3a (males) and 3b (females). Although the number of our cases is small, the presentation is quite detailed, since the data are unique and it seems unlikely that a larger body of similar determinations will be available in the near future. Age of onset of appearance of each of 70 centers is given, for each sex group, in terms of the range, median, and mean. In each instance the number of cases (N), standard deviations (S.D.) and coefficients of relative variability (V) are shown.

TABLE 3a

Male Chimpanzees. Age in Months at Appearance of 70 Centers of Ossification on Left Side of Body

Rank[1] Order	Center[2]	N	Range[3]	Median	Mn.	S.D.	V
3	Head of humerus	5	PN - PN	PN	(—1.0)	(0)	
3	Capitate	5	PN - PN	PN	(—1.0)	(0)	
3	Hamate	5	PN - PN	PN	(—1.0)	(0)	
3	Distal femur	5	PN - PN	PN	(—1.0)	(0)	
3	Cuboid	5	PN - PN	PN	(—1.0)	(0)	
6.5	Distal radius	5	PN - 0	PN	(—0.8)	(.45)	
6.5	Lateral cuneiform	5	PN - 0	PN	(—0.8)	(.45)	
8	Proximal tibia	5	PN - 1.5	PN	(—0.3)	(1.1)	
9	Head of femur	5	0 - 2.5	0	0.7	1.1	157
10	Coracoid*	4	PN - 3	0	(0.5)	1.9	
11	Medial cuneiform	5	0 - 1.5	1	1.0	.6	65
12	Capitulum	5	PN - 2.5	1.5	(1.3)	(1.3)	
13	Distal tibia	5	PN - 3	1.5	(1.4)	(1.6)	
14	Distal fibula	4	2 - 7.5	2.5	3.6	2.6	72
15	Metacarpal 3	6	2.5 - 10	3.8	4.9	2.9	58
16	Triquetral	5	2.5 - 6	4	4.2	1.4	32
17	Greater multangular	6	3.5 - 9	4	5.3	2.2	42
18	Metacarpal 2	6	3 - 10	4	5.5	3.0	54
19	Metacarpal 4	6	3.5 - 13	4	5.9	3.7	63
20	Lunate	5	3.5 - 9	4.5	5.6	2.3	42
21	Proximal radius	6	4 - 13	6.8	7.8	3.6	46

[1]Rank order based on median scores. Where medians are the same, order is determined (a) by upper limit of range when PN scores are involved, (b) otherwise by mean scores.

[2]Centers marked with asterisk are not reported by Pyle and Sontag (9) and are therefore not included in the human-chimpanzee comparisons below.

[3]PN = onset of ossification prenatal; *i.e.*, center present at birth.

TABLE 3a (Continued)
MALE CHIMPANZEES. AGE IN MONTHS AT APPEARANCE OF 70 CENTERS OF OSSIFICATION ON LEFT SIDE OF BODY

Rank[1] Order	Center[2]	N	Range[3]	Median	Mn.	S.D.	V
22	Gt. tuber. humerus	6	2.5 - 17	7.8	7.9	5.0	63
23	Prox. 3rd finger	6	5 - 15	8.5	8.9	3.7	42
24	Navicular (hand)	7	4.5 - 15.5	10	9.7	4.3	45
25	Metacarpal 5	7	6 - 21	10	11.5	5.0	44
26	Prox. 4th finger	8	6 - 17	10.8	10.7	3.3	31
27	Middle cuneiform	8	5 - 16	11	10.4	4.0	38
28	Prox. 3rd toe	7	8 - 17	12	11.6	2.8	24
29	Prox. 4th toe	7	8 - 18	12	12.6	3.3	26
30	Prox. 2nd finger	7	10 - 17	12	12.7	2.2	17
31	Prox. 2nd toe	7	11.5 - 17	12	13.1	2.1	16
32	Distal ulna	8	6 - 28	12	13.1	7.5	57
33	Metatarsal 2	8	8.5 - 23.5	12	13.4	4.8	36
34	Metatarsal 3	8	9 - 25	12.5	13.9	5.1	37
35	Prox. 5th finger	8	10 - 17	13.8	13.4	2.2	17
36	Pisiform*	6	5 - 27	14	14.3	7.6	54
37	Metatarsal 1	8	12 - 18	14	14.5	2.4	16
38	Middle 3rd finger	7	10 - 17.5	15	14.0	3.0	21
39	Middle 4th finger	7	10 - 17	15	14.7	2.2	15
40	Prox. 1st toe	8	14 - 18	15.5	15.7	1.5	10
41	Prox. 5th toe	8	13 - 21	15.5	16	2.5	15
42	Middle 2nd finger	8	11.5 - 18	16.5	16.1	2.4	15
43	Metatarsal 4	8	12 - 27	17	17.3	5.0	29
44	Distal 3rd finger	8	13 - 19.5	17.5	16.8	2.3	14
45	Metacarpal 1	8	10 - 27	17.5	18	6.1	34
46	Middle 5th finger	8	12 - 23.5	17.8	18.1	4.1	23
47	Distal 4th finger	8	13 - 19	18	17.4	2.2	13
48	Proximal fibula	7	16 - 23	18	18.3	2.4	13
49	Metatarsal 5	7	15 - 27	19	20.4	4.8	24
50	Distal 1st toe	8	14 - 27	19.8	20.2	4.0	20
51	Distal 5th finger	8	17 - 27	20	20.9	3.7	18
52	Distal 4th toe	8	18 - 27	20.5	22.3	4.1	18
53	Middle 3rd toe*	8	13 - 23.5	21	19.9	3.3	17
54	Middle 4th toe*	8	13 - 27	21	21.1	4.2	20
55	Distal 2nd finger	8	16 - 26	21.5	21.4	4.0	19
56	Distal 2nd toe	7	20 - 35	22	24.4	5.3	22
57	Distal 3rd toe	8	18 - 30	22.5	23.5	4.2	18
58	Med. epic. humerus	8	15 - 25	23	20.7	4.5	22
59	Lesser troch. femur*	7	18 - 29.5	23	23.1	4.0	17
60	Navicular (foot)	8	16 - 33	24	23.9	5.5	23
61	Distal 5th toe*	7	21 - 30	24	25.6	3.4	13
62	Middle 2nd toe*	8	15 - 29	24.5	23.6	4.5	19
63	Middle 5th toe*	8	14 - 32	24.5	24.1	5.8	24
64	Lesser multangular	7	18 - 39	26	27.9	8.2	29
65	Patella	7	18 - 39	27	26.6	7.5	28
66	Gt. troch. femur	7	24 - 36	27	27.9	4.1	15
67	Prox. 1st finger	8	21 - 36	27	28.9	5.5	19
68	Distal 1st finger	8	21 - 37	29	28.9	6.5	23
69	Trochlea*	7	21 - 38	33	31.3	7.0	22
70	Epiphysis calcaneus	6	32 - 48	42	41.2	6.2	15

TABLE 3b

FEMALE CHIMPANZEES. AGE IN MONTHS AT APPEARANCE OF 70 CENTERS OF OSSIFICATION ON LEFT SIDE OF BODY

Rank[1] Order	Center[2]	N	Range[3]	Median	Mn.	S.D.	V
2	Capitate	7	PN - PN	PN	(—1.0)	(0)	
2	Hamate	7	PN - PN	PN	(—1.0)	(0)	
2	Cuboid	6	PN - PN	PN	(—1.0)	(0)	
5.5	Distal radius	7	PN - 0	PN	(—0.9)	(.4)	
5.5	Head of humerus	6	PN - 0	PN	(—0.8)	(.4)	
5.5	Distal femur	6	PN - 0	PN	(—0.8)	(.4)	
5.5	Lateral cuneiform	6	PN - 0	PN	(—0.8)	(.4)	
8	Head of femur	6	PN - 1	PN	(—0.5)	(.8)	
9.5	Proximal tibia	6	PN - 2	PN	(—0.03)	(1.2)	
9.5	Distal tibia	6	PN - 2	PN	(—0.3)	(1.3)	
11	Coracoid*	4	PN - 1	0	(—0.1)	(1.0)	
12	Capitulum	6	PN - 2	0	(0.3)	(1.0)	
13	Distal fibula	6	PN - 4	0	(0.5)	(2.0)	
14	Medial cuneiform	6	1 - 2	1.5	1.6	0.4	23
15	Gt. tuber. Humerus	6	1 - 9.5	1.5	3	3.3	109
16	Metacarpal 3	6	PN - 2.5	1.8	(1.4)	(1.2)	
17	Distal ulna	7	0 - 16	2	4.9	6.1	125
18	Triquetral	6	1.5 - 4	2.4	2.5	0.9	35
19	Metacarpal 4	6	PN - 3	2.5	(2.1)	(1.7)	
20	Metacarpal 2	6	PN - 4	2.8	(2.3)	(1.7)	
21	Greater multangular	6	2 - 5	3.5	3.4	1.1	33
22	Lunate	6	3 - 7.5	4.3	4.8	1.8	37
23	Prox. 3rd finger	6	1 - 7.5	5.3	4.8	2.3	47
24	Proximal radius	6	2 - 10.5	5.8	5.8	2.8	48
25	Prox. 4th finger	6	2 - 8	6	5.7	2.4	42
26	Navicular (hand)	6	3 - 11	6.8	6.8	2.9	43
27	Metacarpal 5	7	2 - 10	7	6.6	3.0	45
28	Prox. 3rd toe	6	4 - 9.5	7	7.1	1.9	26
29	Pisiform*	7	3 - 19	7	8.6	6.1	71
30	Metacarpal 1	7	5 - 19	7	9.9	5.2	52
31	Prox. 4th toe	7	4 - 9.5	8	7.6	1.9	25
32	Prox. 5th finger	7	3 - 11	8	7.7	2.9	37
33	Prox. 2nd finger	6	2 - 12.5	8	7.8	3.6	46
34	Metatarsal 3	6	3.5 - 11.5	8	7.9	2.9	36
35	Middle 4th finger	7	4.5 - 11	8	8.1	2.4	29
36	Prox. 2nd toe	7	3.5 - 14	8	8.6	3.4	39
37	Metatarsal 2	6	3.5 - 11	9	8.3	2.8	33
38	Middle 3rd finger	7	4.5 - 11	9	8.4	2.6	30
39	Prox. 1st toe	7	8 - 11.5	10	9.6	1.3	13
40	Middle 2nd finger	7	5 - 14	10	10	2.9	29
41	Prox. 5th toe	7	5.5 - 18	11	11.2	4.5	40
42	Metatarsal 1	7	8 - 17	11	11.7	3.0	25
43	Metatarsal 4	7	4 - 18	11	12.1	4.9	40
44	Distal 1st toe	7	8 - 21	11	12.3	4.5	36

[1]Rank order based on median scores. Where medians are the same, order is determined (a) by upper limit of range when PN scores are involved, (b) otherwise by mean scores.

[2]Centers marked with asterisk are not reported by Pyle and Sontag (9) and are therefore not included in the human-chimpanzee comparisons below.

[3]PN = onset of ossification prenatal; *i.e.*, center present at birth.

TABLE 3b (Continued)
FEMALE CHIMPANZEES. AGE IN MONTHS AT APPEARANCE OF 70 CENTERS OF OSSIFICATION ON LEFT SIDE OF BODY

Rank[1] Order	Center[2]	N	Range[3]	Median	Mn.	S.D.	V
45	Middle 5th finger	7	5.5 - 15	12	11.2	3.1	28
46	Distal 3rd finger	7	6 - 15	12	11.3	3.0	26
47	Distal 4th finger	7	7.5 - 16	12	12.2	3.0	25
48	Middle cuneiform	7	11 - 16.5	13	13.2	2.1	16
49	Med. epic. humerus	7	10 - 17	13.5	13.9	2.4	17
50	Middle 3rd toe*	7	11 - 18	14.5	14.2	2.4	17
51	Middle 4th toe*	7	12 - 19	14.5	15.1	2.9	19
52	Metatarsal 5	7	6.5 - 21	15	14.3	4.9	34
53	Distal 2nd finger	7	9 - 19	15	14.9	3.6	24
54	Distal 5th finger	7	9 - 24	15	15.9	4.6	29
55	Proximal fibula	7	9 - 20	16	15.4	3.8	25
56	Lesser troch. femur*	7	13 - 18	17	16.1	1.9	12
57	Middle 2nd toe*	7	13.5 - 21	17	17.1	3.0	17
58	Distal 4th toe	7	14 - 21	18	17.1	2.4	14
59	Distal 3rd toe	7	14.5 - 22	18	17.4	2.6	15
60	Navicular (foot)	7	12 - 28	18	18.4	5.6	31
61	Middle 5th toe*	7	15 - 27	18	19.8	4.1	21
62	Prox. 1st finger	7	10 - 30	18	20	7.9	40
63	Trochlea*	7	11 - 27	19	18.9	6.0	32
64	Distal 5th toe*	7	18 - 20	19	19	0.8	4
65	Patella	7	16 - 24	20	19.9	2.6	13
66	Distal 2nd toe	7	16.5 - 22	20	19.9	1.8	9
67	Distal 1st finger	7	17 - 27	21	21.1	3.7	17
68	Gt. troch. femur	5	16 - 29	21	22	5.1	23
69	Lesser multangular	7	19 - 39.5	21	27.7	9.7	35
70	Epiphysis calcaneus	7	26 - 37	32	31.1	3.8	12

These statistics obviously are of limited applicability and significance in view of the low N's. For those centers which, in some or all subjects, appear prenatally, an arbitrary value of —1.0 month was assigned to "PN" for calculation of the means and S.D.'s.

Individual differences and their reliability

In order to obtain a single, over-all index of acceleration or retardation in postnatal appearance of ossification centers for each animal, a number of statistical procedures are possible. Thus an "average age" might be calculated by taking the mean of the 70 onset-ages for each subject. Since all measures are not available for all animals, however, such means would not be comparable; the omission of late-appearing centers affects the mean differently than the omission of those appearing at an earlier age. A much more representative index is available in terms of Z-scores, which were

computed as follows: For each center, the mean onset-age in months was determined for the group (males and females separately). For each individual, then, this mean was subtracted from his own particular onset-age, and the difference was divided by the S.D. of the group for that center; this quotient is the Z-score. A minus Z-score (the individual's onset-age lower than the mean) indicates acceleration or earlier-than-average onset; a plus sign, contrariwise, indicates relative retardation. The component Z-scores for a given center, of course, add up to zero. An over-all index of ossification-rate—which takes into account differing absolute ages and variabilities—is then obtainable by averaging an individual's several Z-scores (the number varying from 29 for Spratt to 70 for Scarf, Jed, Flora and Karla).[1] The individuals may now be ranked from most accelerated (largest negative mean Z-score) to most retarded (largest positive score), as has been done in tables 4a (males) and 4b (females). The mean Z-scores give a basis, also, for comparison of ossification-rate with various other factors, such as gestation length, birth weight, dentition, and so on.

For any given individual among males and females, respectively, the smallest ranges of Z-scores are —.95 to +1.33 (Spratt) and —.84 to +1.95 (Fanny); the greatest ranges are —2.12 to +2.00 (Jed) and —1.62 to +2.02 (Karla). These figures show that no one center is a safe index of the general acceleration-retardation in ossification rate for an individual. Every animal has some minus and some plus Z-scores. The most "advanced" animals have 58 minus, 7 plus, 5 zero (Scarf) and 62 minus, 4 plus, 3 zero (Falla) scores. For the most retarded animals the figures are: 39 plus,

[1]The circumstance that the nine Z-scores of table 4a do not algebraically add up to zero is a function of the unequal number of measures available for the several subjects. In comparing mean Z-scores we would have had either to limit ourselves to those measures to which *each* member of the group contributed, or to make an assumption regarding the gaps in our data. Since there were *no* N's of 9 (see table 3a), the latter course was chosen. One possible assumption is that the individual is average (Z-score= zero) in respect to those centers on which that individual was not scored. With this assumption, each individual's total Z-score would be divided by 70; the means would then add to zero but the differences between individuals would be obscured. Another assumption, and the one which is here used, is that the mean of the *available* Z-scores is the best indication of the probable Z-scores for the non-scored centers; in this case the mean Z-score is divided by the actual number of measures for each animal (*i.e.*, the N's shown in table 4a). The fact that the average of the nine mean Z-scores differs only slightly from zero (*i.e.*, +.12) suggests that the accepted assumption is approximately correct.

15 minus, 5 zero (Web) and 52 plus, 13 minus, 4 zero (Fanny) scores.

Despite the individual variabilities in Z-scores as reflected in the S.D.'s of tables 4a and 4b, a majority of the differences between the

TABLE 4a
MEANS OF Z-SCORES FOR EACH OF NINE MALE CHIMPANZEES

Rank[1]	Animal	N[2]	Mean Z-score	S.D
1	Scarf	70	—.65	.50
2	Jed	70	—.64	.91
3	Spratt	29	—.26	.49
4	Art	38	—.06	.66
5	Ken	41	—.05	.69
6	Alf	45	+.14	.72
7	Jent	69	+.18	.71
8	Bard	54	+.66	.74
9	Web	59	+.80	1.00

[1]From most accelerated (#1) to most retarded (#9).
[2]Number of measures available, out of possible total of 70.

TABLE 4b
MEANS OF Z-SCORES FOR EACH OF SEVEN FEMALE CHIMPANZEES

Rank[1]	Animal	N[2]	Mean Z-score	S.D
1	Falla	69	—1.02	.67
2	Flora	70	— .38	.55
3	Banka	69	— .27	.61
4	Jenny	44	— .01	.64
5	Karla	70	+ .43	.77
6	Jojo	69	+ .58	.86
7	Fanny	69	+ .66	.74

[1]From most accelerated (#1) to most retarded (#7).
[2]Number of measures available, out of possible total of 70.

mean Z-scores are significant. The reliability of these differences is relevant to our more inclusive survey of individual differences within this group of chimpanzees, and also to the probability of finding relationships between speed of ossification and other growth indices. Critical ratios were therefore computed for all pairs of animals of the same sex (36 male pairs, 21 female pairs). The results may be summarized as follows: Among the males, all 26 differences between means of .38 (Jed-Spratt) and over, yielded critical ratios of 2.7 or more, ranging up to 11.23 (Scarf-Bard). Only 10 of the 36 differences were lower than .38 and these had critical ratios of 1.76 or less. Among the females, the mean difference between Banka

and Jenny is .26, giving a critical ratio of 2.14. The 16 mean differences of .37 or more yield ratios from 3.16 to 14.00, whereas the 4 differences less than .26 give critical ratios of 1.80 down to .59.

In addition to the mean Z-scores and ranks based on all available measures, as given in tables 4a and 4b, we also determined means and their ranks for odd and for even Z-scores. The rank-difference correlations between ranks based on means of odd and even measures, respectively, are +.85 for males, +.96 for females. The shifts of rank according to means based on total, odd, and even measures involve the following pairs of animals: for each of these pairs the critical ratios of the differences between mean total Z-scores (see preceding paragraph) are 1.43 or less: Scarf-Jed; Spratt-Art; Art-Ken; Art-Alf; Ken-Alf; Alf-Jent; Jojo-Fanny.

Variability in short bones and epiphyses

Pyle and Sontag (9) report that in human children "carpal and tarsal bones are definitely more variable in time of appearance than are true epiphyses." The three pertinent bones showing the highest variability in man are the capitate, hamate and lateral cuneiform. These centers usually begin to ossify prenatally in chimpanzee. The rest of the carpals and tarsals of chimpanzee show only slightly greater variability in onset-age than do the epiphyses.

Relations to Other Factors and Measures

Sex differences in order and age of appearance

In tables 3a and 3b, 70 centers of ossification are ranked in order of average age of appearance for the males and females separately. The rank-difference correlation between the two sets is +.97 with a P.E. of .005. The largest rank differences are for the distal ulna and metacarpal 1 (females relatively advanced) and for the middle cuneiform and distal 2nd toe (males relatively advanced).

The median of the 70 median ages for males is 13.9, for females 8.0 months. The means of the 70 means for each sex show a smaller difference but in the same direction: 13.76 with an S.D. of 9.77 for males, 9.48 with an S.D. of 7.66 for females. The mean difference of 4.28 between the sex groups is significant at beyond the .0001 level of confidence. The seventy differences between male and female means are plotted in Figure 1. Table 5 shows the number of cases

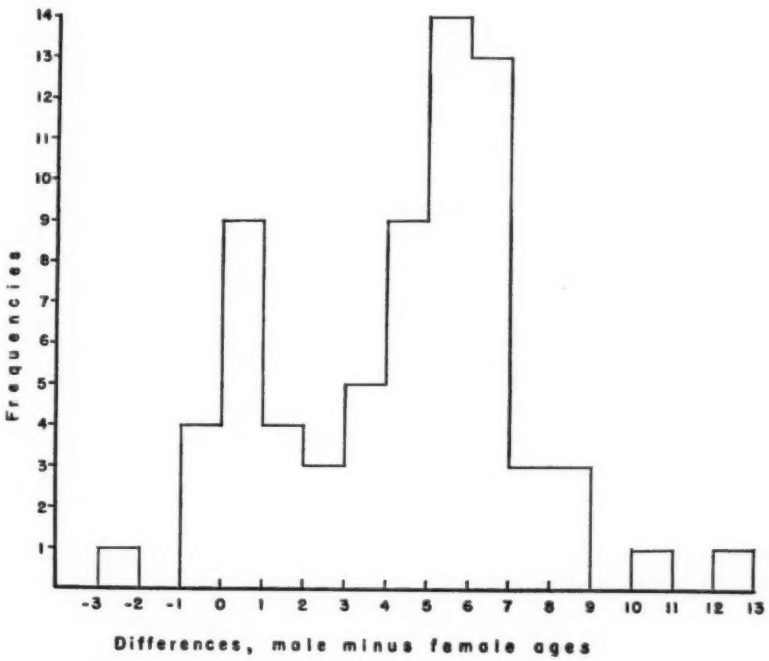

Figure 1. Distribution of 70 differences between mean onset ages for male and female chimpanzees. Figures along the abscissa represent the *beginning* of a step-interval; the 0 to +1 interval includes 4 cases of no difference.

TABLE 5

	According to medians	According to means
Number of centers for which male age equals female age	9	4
Number of centers for which male age is higher	59	61
Number of centers for which male age is lower	2	5

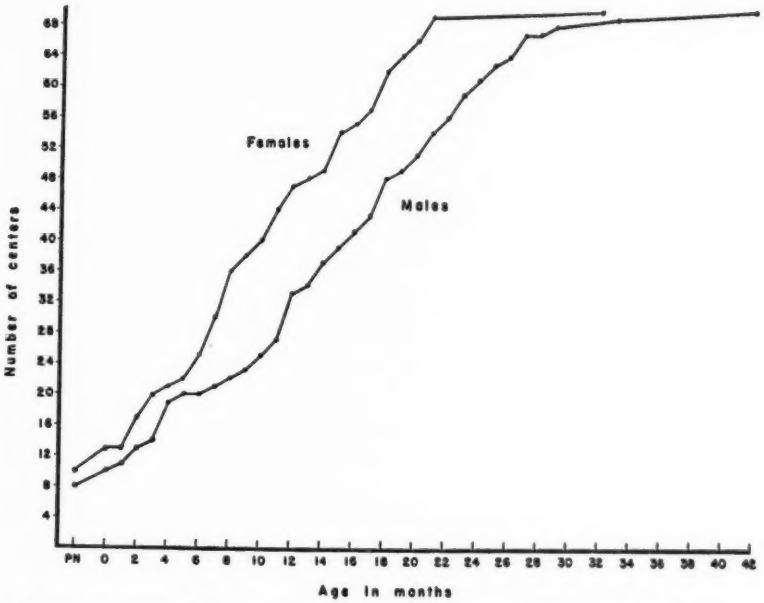

Figure 2. Cumulative curves showing median number of ossification centers present at successive one-month intervals in male and female chimpanzees. These curves are based on the data presented in tables 3a and 3b. The number of centers with prenatal onset is indicated at "PN"; birth is shown as age zero.

in which the median and mean ossification ages of the males are equal, higher, and lower than those of the females.

The difference between our 9 male and 7 female chimpanzees is further illustrated in Figure 2, (which is based on medians rather than means). It shows that at each age a larger number of centers has begun to ossify in the median female than in the median male. These curves show cumulative totals, and do not differentiate among the 70 centers under consideration.

Individual variability within each sex group may be compared by calculating the mean coefficient of variability of 54 comparable centers—i.e., those having no PN score in either group. The mean for males is 26.4, for the females it is 32.4.

Relations to length of gestation and birth weight[2]

As a "zero point" for growth of the individual, the moment of conception has obvious advantages over any other arbitrarily designated occurrence, such as that of birth. This is particularly clear in respect to the onset of ossification in centers which have already attained considerable size when the animal is born. On the assumption that the appearance of ossification centers is primarily a function of time elapsing since conception, a high positive correlation would be expected between birth-age and acceleration in ossification-age. According to this view Jent, born 238 days after conception, should have attained about the same stage at birth as that reached at the birth-age of one month by Bard who had only 207 days of intra-uterine life. This, of course, assumes an equivalence between intra-uterine and postnatal time in its effect on growth.

Rank-difference correlations have been computed between relative acceleration-retardation in appearance of 70 ossification centers (Z-score ranks given in tables 4a and 4b) and length of gestation (data from Nissen and Yerkes, 8), the longest period being given the lowest rank. These coefficients are + .18 for the males, —. 17 for the females. There is, thus, no statistical support for the expectation outlined above.

If the figures are examined in greater detail, it is seen that gestation lengths among the females are rather closely and evenly spaced in the range of 220 to 241 days. Among the males, however, there are two animals with definitely shorter gestations (Bard 207 days, Web 204 days) than the other seven males (222 to 236 days). Bard and Web are also the most retarded males in respect to mean ossification Z-score (i.e. ranks 8 and 9, respectively). This suggests that the effects of small variations in gestation lengths are obscured by other factors, but that extreme variation is accompanied by a corresponding effect on ossification-age as measured from birth.

[2]On *a priori* grounds, a direct or inverse correspondence between ossification rate and such factors as gestation length, birth weight, dentition, and postural development, would not appear improbable. A rank-difference correlation coefficient of plus or minus 1.0 within each sex group would strongly indicate such correspondence. In view of 1) the small number of subjects and 2) the limited reliability of some of the measures involved, however, extreme caution is necessary in interpreting any coefficients smaller than one as indicating either presence or absence of a relationship between the measures under consideration. Within the limits of reliability, a less-than-perfect correlation does indicate that one variable is not the sole determinant of the other, and that both variables are not completely determined by a common factor.

Using only the data from the 5 males and 5 females for whom complete sets of birth X-rays are available, rank-order correlations were computed between gestation length and the number of centers (a) ossified prenatally or (b) present at birth (PN plus O). The coefficients are +.67 and +.57 for the males, —.68 and —.38 for the females. These figures again suggest that differences in ossification rate are a function of both time and of factors specific to the individual organism. The fact that there is a rank-order correlation of +.60 for males and +.61 for females between birth weight (see table 1) and mean ossification rate (tables 4a and 4b) suggests a possible relationship between hereditary or intra-uterine factors controlling ossification rate and growth as measured by weight.

Relations to dentition and to postural development

Data on eruption of the deciduous teeth in the 9 male and 7 female chimpanzees of this study are available (table 2, Nissen and Riesen, 7). The rank-difference correlations between average age at eruption of the 20 deciduous teeth and the Z-score ranks of tables 4a and 4b are +.77 for males, —.68 for females. Positive and negative correlations are perhaps equally plausible, the former ascribable to a common determining mechanism, the latter to "compensatory" or "competitive" mechanisms. However, there is no basis for assuming that one of these factors would operate predominately in the case of males, the other one in females. The present results, therefore, justify only the statement that no consistent evidence has been obtained—for the population-sample of male and female chimpanzee —indicating a relationship between speed of (a) deciduous dentition and (b) onset of ossification in the epiphyses and short bones of the extremities.

Equally ambiguous results appear when ossification age is related to postural development as measured by the Gesell scale. Riesen and Kinder (10) report test results on 5 of the males and on all 7 of the female subjects of the present study, and these have been ranked within each sex from most advanced to most retarded in 60 postural-activity items. Rank-difference correlations with our Z-score ratings give coefficients of +.20 for the 5 males, —.61 for the 7 females.

Comparisons with Human Data

The comparisons made in this section are based on the human data presented by Pyle and Sontag (9) and the chimpanzee data given in tables 3a and 3b of this report. Since the former comprise 61 (instead of 70) centers, comparison figures for the male and female chimpanzee groups are limited to the same 61 centers. In those calculations involving means, standard deviations and coefficients of relative variability, all centers having one or more PN (prenatal onset) scores for the chimpanzee subjects were omitted for both chimpanzee and human groups.

Order of appearance of individual centers

The rank-difference correlation coefficient between order of appearance of 61 ossification centers in man and chimpanzee is +.70 for the male groups, +.68 for the females. In table 6 are listed those centers for which the rank-order difference between chimpanzees and human subjects is 9 steps or more in either male or female groups. A plus sign indicates that the center appears relatively early

TABLE 6

Ossification Centers with Large Difference between Man and Chimpanzee in Relative Order of Appearance

Figures show rank in human minus rank in chimpanzee group.

I. *Appearing relatively earlier in chimpanzee*	Diff. in rank		II. *Appearing relatively later in chimpanzee*	Diff. in rank	
	Males	Females		Males	Females
Distal ulna	+29	+44	Distal 1st finger	—41	—39
Greater multangular	+41	+36	Distal 1st toe	—33	—28
Prox. radius	+36	+34	Prox. 1st finger	—18	—17
Navicular (hand)	+32	+33	Navicular (foot)	—16	—12
Lunate	+28	+30	Prox. 2nd finger	—13	—15
Triquetral	+16	+22	Distal 4th finger	—11	—14
Medial cuneiform	+13	+15	Gt. troch. femur	—12	—13
Metatarsal 3	+12	+13	Distal 3rd finger	— 9	—11
Distal radius	+ 6.5	+ 9.5	Middle 3rd finger	— 9	—10
Metacarpal 4	+ 8	+ 9	Prox. 2nd toe	— 5	—12
Metatarsal 4	+ 8	+ 9	Gt. tuber. humerus	—10	— 3
			Prox. 5th finger	—10	— 6
			Middle 2nd finger	—10	— 8
Middle cuneiform	+ 9	—10	Middle 4th finger	— 9	— 8
			Distal 2nd finger	— 9	— 5
			Prox. 3rd finger	— 8	— 9
			Prox. 3rd toe	— 7	— 9
			Distal 5th finger	— 6	— 9

in chimpanzee (rank of center in man is higher—i.e. later—than in ape), whereas a minus sign indicates that it appears relatively early in man. It will be noted that the sign of the rank-order difference is the same for males and females in all cases except one: the middle cuneiform. In man this center has almost the same rank for boys and girls (No. 35 and No. 36, respectively), whereas among chimpanzees this center shows the largest rank-order difference between males and females: No. 26 and No. 46, respectively. The discrepancy, therefore, may be attributed to an apparent sex difference, in regard to the middle cuneiform, in our samples of male and female chimpanzees.

No detailed interpretation of these comparisons will be attempted here. It may be noted, however, that in general the chimpanzee is relatively advanced with respect to those centers of the extremities —particularly of the forelimb—concerned with weight-bearing and locomotor functions. The human children, on the other hand, are relatively advanced in ossification of epiphyses of the phalanges, particularly of the hand. It is tempting to relate this acceleration to the earlier and more fully developed manual, especially prehensory, activities of man. It should be noted that the phalanges may also serve "weight-bearing and locomotor functions" in gross clasping and climbing. In these latter activities, however, the contractile and tensile strength of muscles and tendons is chiefly involved, whereas in standing, walking, and running rigidity of the limbs may be aided by growth of the long-bone epiphyses. Control of fine movements of manipulation may likewise be assisted by development of the phalangeal epiphyses, inasmuch as this contributes towards fixation (see Jones, 5) and a more precise determination of the fulcrum of the joints of the fingers (and toes).

Absolute age at appearance of centers

The medians of the 61 mean ages for human boys and girls (Pyle and Sontag, 9) are 27.3 and 19.9 months, respectively. The medians of the corresponding 61 medians of male and female chimpanzees are 12.0 and 8.0 months, respectively (rank 32 in table 3a; rank 33 in table 3b). On this basis of comparison the species difference is 15.3 months in the case of males, 11.9 months for females. Using, instead, the median of the 61 chimpanzee means, these differences become 14.2 and 12.0 months, respectively.

Comparable mean ossification ages for the two species may be computed by eliminating from both human and chimpanzee data (a) the 9 centers not included in the former and (b) those centers involving one or more PN scores for the chimpanzees. This leaves 51 comparable means for the human and chimpanzee males, 46 comparable means for the female groups. The differences between means are plotted in Figures 3 and 4. The resulting means of means are

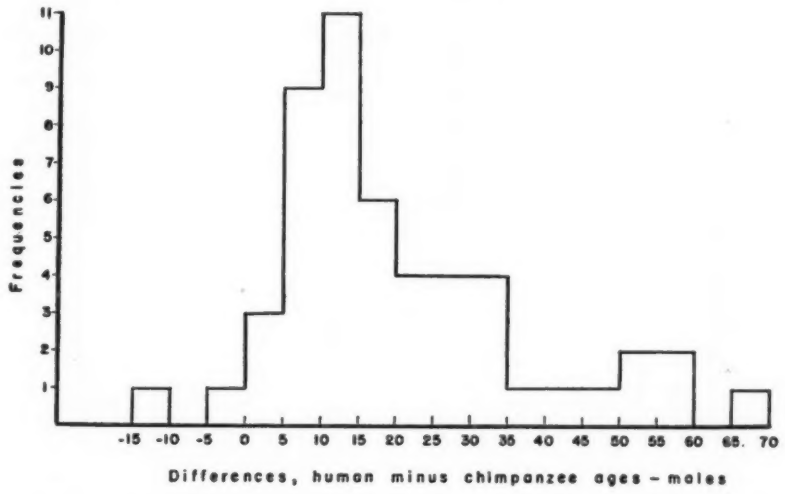

Figure 3. Distribution of 51 differences between mean onset ages for human and chimpanzee males.

shown in table 7. These differences between the comparison groups are significant at a very high level of confidence. That the finding may be generalized as reflecting a species difference—rather than merely a difference between our samples of the two populations—is indicated by the following: (a) Of the 51 male comparisons, the human mean ages are higher than those of chimpanzee in 49 cases and in 47 of these the critical ratio (difference/S.D. $_{diff.}$ is 3 or higher. (b) In 44 of the 46 comparisons among females, the human mean ages are higher than those of the chimpanzees; of the 44 all but 4 mean differences have a critical ratio exceeding 3. The two centers for which mean ossification age is lower in man are, in males

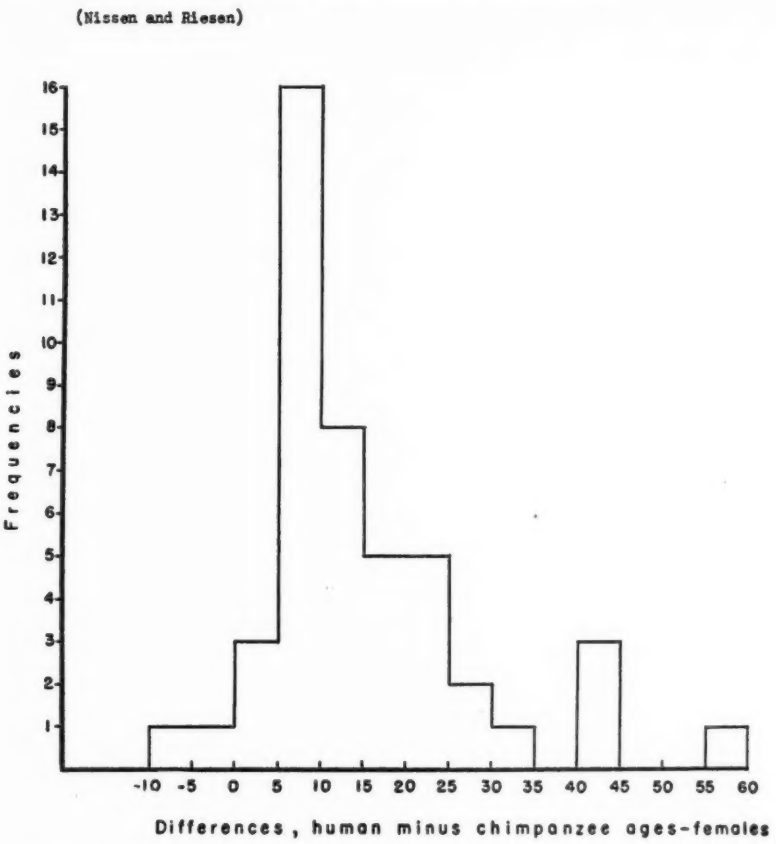

Figure 4. Distribution of 46 differences between mean onset ages for human and chimpanzee females.

and females, the epiphyses of the first distal toe and of the first distal finger.[3]

[3]The centers used for comparison of mean differences may be identified in tables 3a and 3b from the criteria stipulated in this paragraph. The centers with statistically unreliable differences (critical ratios in parentheses) are, for the males: greater tubercle of the humerus (1.5) and proximal first finger (2.6); for the females they are: proximal first finger (0.5), proximal second finger (2.1), greater tubercle of the humerus (2.5), navicular of the foot (2.8).

TABLE 7
MEAN OSSIFICATION AGES AND COEFFICIENTS OF VARIABILITY FOR HUMAN AND CHIMPANZEE GROUPS
Human means and V's calculated from data of Pyle and Sontag (9).

	Males		Females	
	Mn. of 51 mn. ages	Mn. of 51 V's	Mn. of 46 mn. ages	Mn. of 46 V's
Human subjects (N = 31 to 50)	35.8 mos.	27.8	26.3	28.8
Chimpanzee (N = 4 to 8)	15.4	32.4	11.6	33.8
Difference	20.4	4.6	14.7	5.0

Variability

The somewhat higher mean coefficients of relative variability for the chimpanzees as compared to the human subjects (table 7) may represent a species distinction, but it will be noted that the differences are small.

Sex differences

In both species the males are considerably slower than the females in average age at which the various ossification centers are first seen on roentgenograms. In terms of the median of 61 measures, the sex difference is 7.4 months in man, 4 months in chimpanzee—a male/female quotient of 1.37 in the former, 1.5 in the latter species. Various other statistical bases of comparison give similar differences and ratios.

Relative age-differences for six regions of the extremities

Further light may be thrown on relative acceleration-retardation in various parts of the body, in man and chimpanzee, by combining the centers belonging to certain anatomical (and functional) areas. Thus, the 61 comparable centers may be grouped into the following six regions: arm (epiphyses of humerus, radius, ulna), wrist (carpals), hand (epiphyses of metacarpals and fingers), leg (epiphyses of femur, tibia and fibula, and the patella), ankle (tarsals), and foot (epiphyses of metatarsals and toes). For present purposes, the two sex groups within each species may be combined. The mean age (of the mean ages) for each region may then be computed for man and for chimpanzee; results are shown in table 8.

TABLE 8

MEAN OSSIFICATION AGES IN MAN AND CHIMPANZEE FOR CENTERS GROUPED INTO SIX REGIONS

(Each figure—other than the ranks—is the mean of the mean ages, for males and females, which are given in tables 3a and 3b and by Pyle and Sontag (9). Means with minus values for the chimpanzees, *i.e.*, involving cases of prenatal onset, are included.)

	Arm	Wrist	Hand	Leg	Ankle	Foot
Number of centers	7	7	19	8	6	14
Human mean age	30.4	33.9	21.6	17.3	25.8	30.2
Chimpanzee mean age	5.3	6.7	12.4	8.3	11.4	14.7
Differences	25.1	27.2	9.2	9.0	14.4	15.5
Human ranks in age	5*	6	2	1	3	4*
Chimpanzee ranks in age	1	2	5*	3	4*	6
	Upper extremity			Lower extremity		
Human mean age	26.1			25.6		
Chimpanzee mean age	9.7			12.0		

*There is a reversal of these two ranks for one sex group when data of the two sex groups are computed separately.

These figures are in harmony with the previous conclusion based on order of appearance among individual centers: In chimpanzee, the weight-bearing arm and wrist regions ossify before the area of the manipulating hand; in man this order is reversed. In man, the weight-bearing leg is even earlier than the hand, whereas in chimpanzee the arm, wrist, leg and ankle regions are all more advanced than hand and foot. The acceleration of the upper, as compared with the lower extremity in chimpanzee, is largely a function of advancement in the arm and wrist areas; in man there is almost no diffierence in mean ages of the upper and lower extremities.

SUMMARY

Serial roentgenograms were made of nine male and seven female chimpanzees at two or three month intervals from birth to the age of four years. These records were used to determine the ages at which each of 70 epiphyses and short bones of the left extremities first begin to ossify.

The basic data for males and females are presented in tables showing measures of central tendency, variability, and order of appearance of the 70 centers. Mean standard scores were computed for each animal to permit the assignment of rank-orders in "ossification age" and comparison of this index with other known measures and factors pertaining to the same individuals.

The centers under consideration begin to ossify, on the average, about five months earlier in females than in males. The rank-difference correlation in order of appearance in the two sexes is +.97. Extremely short gestation periods are accompanied by retardation (as measured from birth) in average age at appearance of the centers; moderate variations in gestation length appear to be unrelated to mean ossification age. A rank-order correlation of +.60 was found with birth weight, suggesting a possible relationship between genetic or intra-uterine factors controlling ossification rate and growth as measured by weight. No consistent relations were found with either postural development or eruption of the deciduous teeth.

The average age at onset of ossification in epiphyseal and short-bone centers is from 12 to 20 months earlier in chimpanzees than in man. The mean age differences are highly significant for both male and female groups, and in the majority of cases the differences are statistically reliable for the centers considered separately. Among both males and females, however, epiphyses of the distal thumb and great toe begin ossification earlier in man than in chimpanzee. The average acceleration of females over males is relatively about the same in the two species. The rank-difference correlation in order of appearance in the two species is +.70 for males, +.68 for females. Chimpanzees are relatively earlier in those centers, especially of the forelimb, related to weight-bearing and locomotor functions, whereas the human children are relatively advanced in ossification of epiphyses of the phalanges, particularly of the hand.

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